IMMUNOLOGICAL STUDIES IN ACUTE LEUKEMIA AND EFFECT OF IMMUNE COMPLEXES ON BLOOD CELLS DETECTED BY MICROCALORIMETRY

by

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Abstract
Cytotoxic antibodies against autochthonous leukemic cells in AML were studied by use of ^{51}Cr release techniques. Assays for (a) complement-dependent cytotoxicity and (b) antibody-associated cellular cytotoxicity (AACC) showed cytotoxic antibodies in sequential sera in all patients studied. In most patients antibody activity was low or absent at the time of diagnosis but appeared after chemotherapy also in patients who did not enter remission. Antibody activity was higher in remission patients when tested in the AACC assay (as compared to patients in whom remission was not achieved). Lack of reactivity with autochthonous remission cells indicates specificity against leukemia-associated antigens (LAA). Furthermore, cytotoxic antibodies were detected in sera of untreated AML patients using a method known to split antigen-antibody complexes by ultrafiltration at low pH in combination with the complement-dependent cytotoxicity assay (see above, a), indicating that potentially cytotoxic antibodies to LAA are bound to soluble antigens forming antigen-antibody complexes.
A ^{125}I protein A binding assay was established to detect also non-cytotoxic antibodies in patients with AML and ALL. Antibodies capable of binding to autochthonous leukemic cells were demonstrated in both types of leukemia after successful chemotherapy. These results were similar to those obtained with the cytotoxic techniques (see above, a and b) and no significant binding to autochthonous remission cells was demonstrated.
A calorimetric method was used to study effect of preformed immune complexes (IC) on human blood cells. Maximal heat production was obtained with IC containing antigens and antibodies at the so called zone of equivalence. Granulocytes were found to be responsible for the main heat production in whole blood after admixture of IC. IC composed of antigen and $F(ab)_2$ fragments prepared from the specific antibodies initiated heat production in the cells only in the presence of active complement, indicating C3b generation through the alternative pathway of complement activation and subsequent binding of C3b to receptors on the cells.

Key words
Acute leukemia, specific antibodies, immune complexes, calorimetry, heat production, complement, complement activation

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The present summary is based on the following papers, which will be referred to by their roman numerals:

I Fäldt R and Ankerst J: Tumor-associated humoral cytotoxicity in patients with acute myelogenous leukemia before and after chemotherapy. Int J Cancer, 20, 824-833, 1977.

II Fäldt R and Ankerst J: Demonstration of antibody-associated cellular cytotoxicity in patients with acute myelogenous leukemia before and after chemotherapy. Int J Cancer, 24, 17-26, 1979.

III Fäldt R and Ankerst J: Possibly specific immune complexes in sera of patients with untreated acute myelogenous leukemia. Int J Cancer, 26, 309-314, 1980.

IV Fäldt R and Ankerst J: A ^{125}I protein A binding assay detecting cell surface antigens: Evidence for the presence of specific antibodies against leukemia-associated antigens in human leukemias.
Submitted for publication, 1982.

V Monti M, Fäldt R, Ankerst J and Wadsö I: A new approach to detection of antigen-antibody complexes by micro-calorimetric measurements of heat production in blood cells. J Immunol Meths, 37, 29-37, 1980.

VI Fäldt R, Ankerst J, Monti M and Wadsö I: Heat production in different populations of human blood cells exposed to immune complexes in vitro: the importance of the Fc parts of immunoglobulins and the influence of active complement. Immunology, 46, 189-198, 1982.

ABBREVIATIONS

AACC	antibody-associated cellular cytotoxicity
ADCC	antibody-dependent cellular cytotoxicity
ALL	acute lymphoblastic leukemia
AML	acute myelogenous leukemia
BCG	bacillus Calmette-Guerin
cALL	subgroup of ALL, "common type"
CALLA	common ALL antigen
C3b	a cleavage product of complement factor C3
C1q	subcomponent of the first complement factor C1
CIC	circulating immune complex
CLL	chronic lymphatic leukemia
CML	chronic myeloid leukemia
ELISA	enzyme linked immunosorbent activity
$F(ab)_2$	antigen binding part of immunoglobulin
FACS	fluorescence activated cell sorter
Fc	fragment crystallizing (part of immunoglobulin)
HMPS	hexose monophosphate shunt (aerobic glucose catabolism)
HTLV	human T cell leukemia-lymphoma virus
IC	immune complex
LAA	leukemia-associated antigen
LANA	leukemia-associated nuclear antigen
M1 - M5	French-American-British classification of AML
MLC	mixed leukocyte culture test
Raji-cell	B lymphocyte in vitro cell line originally derived from a patient with Burkitt lymphoma
SLE	systemic lupus erythematosus

CONTENTS

PART ONE - ANTIBODIES TO CELL SURFACE ANTIGENS IN ACUTE LEUKEMIA

INTRODUCTION....3

ANTIGENS PRESENT ON LEUKEMIC CELLS

1. HLA-antigens....5
2. Immune associated antigens....5
3. Differentiation antigens....6
4. Leukemia-associated antigens....7
5. Virus-related antigens....9

BACKGROUND TO THE PRESENT STUDY

A. Cell-mediated immunity to acute leukemia

1. Mixed leukocyte culture tests....10
2. Lymphocyte cytotoxicity....11
3. Cutaneous delayed hypersensitivity....12

B. Humoral immunity to acute leukemia

1. Complement-dependent cytotoxicity....12
2. Antibody-dependent cellular cytotoxicity....14
3. Cytophilic antibodies....14
4. Antibodies detected by immunofluorescence....14

THE PRESENT INVESTIGATION

Paper I....16
Paper II....17
Paper III....18
Paper IV....19

GENERAL DISCUSSION....20

MAIN FINDINGS IN THE PRESENT STUDY....29

REFERENCES....31

CONTENTS continued

PART TWO - HEAT PRODUCTION INDUCED BY THE INTERACTION OF IMMUNE COMPLEXES WITH NORMAL BLOOD CELLS

INTRODUCTION.....40

BACKGROUND TO THE PRESENT STUDY

1. Circulating immune complexes.....41
2. Interaction of immune complexes with blood cells.....42
3. Metabolic activation induced by immune complexes.....43

THE PRESENT INVESTIGATION

Paper V.....44
Paper VI.....45

GENERAL DISCUSSION.....45

MAIN FINDINGS IN THE PRESENT STUDY.....49

REFERENCES.....50

ACKNOWLEDGEMENTS.....56

PAPER I

PAPER II

PAPER III

PAPER IV

PAPER V

PAPER VI

PART ONE - ANTIBODIES TO CELL SURFACE ANTIGENS IN ACUTE LEUKEMIA

INTRODUCTION

During the past decade much effort has been spent to demonstrate the presence of leukemia-associated antigens (LAA) and specific cell-mediated and humoral immune responses to such antigens. Heteroantisera with more or less restricted specificities have been produced through immunization of different animal species. Recently monoclonal hybridoma antibodies have been developed which are claimed to be specific for antigens associated with some subgroups of acute lymphoblastic leukemia (ALL). Most studies to detect LAA in acute myelogenous leukemia (AML) and ALL by cell-mediated and humoral assays have been performed with allogeneic combinations of cells and sera. Therefore the specificity of such reactions is difficult to ascertain because of the variety of antigenic systems represented both on normal blood cells and leukemic cells. Weak reactions to such antigens can be interpreted erroneously as leukemia specific. However, some cell-mediated immune reactions have been studied in autochthonous systems and strongly suggested specificity to LAA in acute leukemias. At the time of the start of the present studies only very few reports on the presence of humoral antibodies against LAA on autochthonous cells had been published. Therefore we decided to develop new techniques which could be used to detect antibodies against LAA in autochthonous systems, thereby avoiding reactions against not relevant antigens. Since it is known that cytostatic drugs used in the treatment of acute leukemia can be immunosuppressive in high doses, our intention was also to look for these antibodies before as well as during and after conventional chemotherapy. Circulating immune complexes (CIC) have been shown in numerous reports to be more prevalent in the acute and relapse stages of acute leukemia. This led us also to study whether such complexes in sera of untreated patients with AML may contain soluble LAA and specific antibodies.

ANTIGENS PRESENT ON LEUKEMIC CELLS

There are several groups of antigens associated with leukemic cells and the majority of them are also present on normal blood cells or their precursors. Antigens coded for by genes of the HLA-A, HLA-B and HLA-C loci within the major histocompatibility complex of chromosome 6 are expressed on leukemic cells as well as on normal nucleated cells. The genes of the HLA-D region control the expression of antigens that appear to be analogous to murine immune-associated (Ia) antigens. They are serologically detectable on some normal blood and bone marrow cells and on most leukemic cells. Normal and leukemic cells express several so called differentiation antigens which are linked to certain stages of cellular maturation and to certain cell types. These antigens are serologically detectable by heteroantisera or monoclonal antibodies and should more appropriately be called markers because they are probably not immunogenic in autochthonous systems. Distinct from these normally expressed structures are the leukemia-associated antigens (LAA), which probably are qualitatively new antigens formed by unknown mechanisms. Theoretically LAA may be formed as a result of virus infection or genetic mutations and/or rearrangements secondary to exposure to chemicals, radiation or other factors.
The following antigen systems represented on leukemic cells will be discussed more in detail:

1. HLA-antigens (HLA-A, B and C antigens)
2. Immune-associated antigens (Ia)
3. Differentiation antigens (markers)
4. Leukemia-associated antigens (LAA)
5. Virus-related antigens.

1. HLA-antigens

The HLA-A, B and C antigens are expressed on leukemic cells as well as on normal nucleated cells. It has been reported by Pegrum et al (1970) that leukemic cells may acquire new HLA-antigens. Harris and Viza (1971) suggested that anomalous HLA typing results with leukemic cells are due to contamination of HLA antisera with antibodies too weak to be detected by cytotoxic tests employing normal cells. They found that AML blasts are more sensitive to HLA antisera. Thus both differences between normal and leukemic cells in susceptibility to testing and the sensitivity of the assay used may explain somewhat differing results when testing leukemic cells, remission cells and normal cells for the presence of HLA-antigens. It is important to consider weak reactions to HLA-antigens when interpreting results obtained in allogeneic combinations testing leukemic cells and leukemic or normal sera.

2. Immune-associated antigens

Human Ia antigens can be demonstrated on the surface of various cells using human alloantisera (Winchester et al, 1975), rabbit heteroantisera (Schlossman et al, 1976), murine alloantisera (Ing et al, 1979; Delovitch and Falk, 1979) and mouse monoclonal antibodies (Brodsky et al, 1979). Ia antigens are detectable on various normal cells such as B lymphocytes, monocytes, macrophages, activated T lymphocytes, undifferentiated bone marrow stem cells and a subset of null cells (lacking conventional B and T cell markers). Ia antigens have been identified on all acute leukemia cells with the exception of acute promyelocytic leukemia and T cell ALL. Most chronic myeloid and lymphoid leukemia cells are also Ia positive. According to Mohanakumar and Raney (1978) several of the allogeneic sera claimed to be leukemia specific are directed against Ia antigens.

3. Differentiation antigens

These antigens should be denoted markers because they are probably not immunogenic in the host or in allogeneic systems. They are serologically detected by heteroantisera or monoclonal hybridoma antibodies using the hybridoma technique described by Köhler and Milstein (1975). These markers are linked to certain stages of cellular maturation and to certain histologically defined cell types. Embryonic antigens can be included under this heading because these antigens (markers) are represented on normal cells in some stages of embryonic differentiation. The presence of antigens in sera of patients with acute leukemia and in fetal sera detected by rabbit antisera raised by immunization with solubilized material of acute leukemia cells has been reported by Harris et al (1971). Granatek et al (1976) reported that sera from mice immunized with leukemic blasts reacted with syngenic fetal liver but not with adult liver or bone marrow cells. It should be noted that some embryonic antigens expressed by some experimental neoplasms may be immunogenic in the primary host but up to now there is no evidence for immunogenity of embryonic antigens in the leukemia patient. Roberts and Greaves (1978) raised a rabbit antiserum against myelomonocytic leukemia cells which was reactive with normal granulocytes and their precursors, monocytes and myeloid leukemia cells. The antigen detected by this antiserum, called anti-M serum, was not expressed on lymphocytes, lymphoid leukemia cells or cells in the erythroid series. They also noted that the antigen density increases with increased cellular maturation. With monoclonal hybridoma antibodies cell markers linked to cell surface antigens and related to cellular differentiation have been detected predominantly on normal and leukemic lymphoid cells (Reinherz et al, 1980). However, during the last two years a great number of monoclonal antibodies detecting differentiation antigens on granulocytes, monocytes and their precursors have been presented. Majdic et al (1981) have presented the D5 monoclonal

antibody reactive with granulocytes and acute meylogenous leukemia cells of types M1 - M5 (French-American-British classification) but nonreactive with monocytes. The D5D6 monoclonal antibody produced by Linker-Isreali et al (1981) is reactive with monocytes and acute myelogenous leukemia cells of types M1 - M5 but nonreactive with mature granulocytes and chronic myeloid leukemia cells. This antibody also inhibited the growth of CFU-C, the myeloid-monocytic stem cell. The MY3, MY4, MY7 and MY8 monoclonal antibodies produced by Griffin et al (1981) allow the distinction between granulocytes and monocytes and besides have different specificities to acute myelogenous leukemia cells reacting with cells of the M1 - M5 or M4 - M5 subgroups of the disease.

To summarize ,the heteroantisera raised against differentiation antigens (markers) and the monoclonal antibodies cited are not detecting antigens or markers specific for leukemic cells. They are probably not immunogenic in autochthonous systems and consequently they are not involved in antitumor responses of leukemia patients. However, they can be used to effectively discriminate between different types of acute leukemia, which is important in the evaluation of prognosis and choice of therapy.

4. Leukemia-associated antigens

Leukemia-associated antigens (LAA) are probably distinct from normally expressed molecules. Some of them have been claimed to be specific for leukemic cells while others probably represents a greatly increased expression of certain molecules also present in some normal cells. LAA have been analysed predominantly with heterologous appropriately absorbed antisera raised against whole leukemic cells or leukemic cell membrane preparations. A number of reports describe antigens specific for acute myelogenous leukemia cells detected by mouse (Baker and Taub, 1976; Baker et al, 1979), rabbit (Billing et al, 1980) simian (Metzgar et al, 1972; Mohanakumar et al, 1976) and human (Baker et al, 1978) antisera prepared by immunization with whole leukemic cells.

However, it is apparent that they express more than one specificity and that at least some of the antigens detected by the antisera are restricted to certain histological types of leukemia. Thus, antisera raised in non-human primates detect antigens common to AML and CML on one side and ALL and CLL on the other. By immunization of rabbits with leukemic cells from the common type of acute lymphoblastic leukemia (cALL) antisera reactive with cells from patients with this type of leukemia but non-reactive with AML leukemic cells have been produced (Greaves et al, 1975; Pesando et al, 1979). The antigen detected by these antisera has been designated cALL antigen or CALLA.

Using membrane preparations of AML leukemic cells (Al-Rammahy et al, 1980) and material of membrane extracts obtained after polyacrylamide gel electrophoresis (Malcolm et al, 1982) for immunization of rabbits, strong antisera reactive with AML and CML leukemic cells were found in an enzyme linked immunosorbent assay (ELISA). These antisera did not react with lymphoid leukemic cells, normal leukocytes or bone marrow cells of healthy donors. Malcolm et al (1982) also used this antiserum in the fluorescence-activated cell sorter (FACS) and registered the same specificity as in ELISA. A strong antiserum was produced without any prior absorption by immunization of rabbits with membrane material from the T cell line MOLT-4 (Negoro and Seon, 1981). This antiserum showed specificity for human thymus leukemia-associated antigen (HTL-antigen).

Monoclonal hybridoma antibodies have been mostly used for detection of cell surface markers and differentiation antigens on lymphoid and recently also on myeloid cells. However, data describing antibodies to unique leukemia- or lymphoma-associated antigens have also been presented. One of these monoclonal antibodies is the monoclonal antibody reacting with leukemic cells from patients with cALL (Ritz et al, 1980) which demonstrated lack of reactivity with normal bone marrow cells or fetal hematopoetic tissues in contrast to the heteroantisera detecting the cALL antigen (CALLA) (Greaves et al, 1975; Pesando et al, 1979).

5. Virus-related antigens

It has long been known that RNA type C viruses (retroviruses) can induce a variety of neoplastic tumors such as leukemias, lymphomas and sarcomas in animals. This led to search for evidence of viruses and virus-related antigens in human acute leukemias. The results of these investigations were initially the detection of RNA-directed DNA polymerase (reverse transcriptase) and 70S virus-related RNA in human acute leukemia cells as reviewed by Gallo et al (1974). Viola et al (1976) found the same viral "fingerprints" in remission leukocytes of patients with acute leukemias. Several attempts have been made to detect virus-related "new" antigens on the surface of human acute leukemia cells. Mann et al (1974) who prepared rabbit antisera reactive with AML and ALL leukemic cells by immunization with membrane components from the Raji B lymphocyte cell line, reported that these antisera reacted with embryonic human kidney infected with Rauscher, Kirsten or SV-40 viruses. Mohanakumar and Raney (1978) reported relationships between membrane antigens on human leukemic cells and RNA virus antigens. They found that goat antiserum to Friend leukemia virus (FLV) was cytotoxic to cells from all AML, CML and 3/5 ALL patients. Recent results strongly implicate an etiologic association between a newly discovered human type C retrovirus, human T cell leukemia-lymphoma virus (HTLV), with certain types of T cell leukemias and lymphomas as reviewed by Gallo and Wong-Staal (1982). Clusters of this form of T cell neoplasia have been found in South-Western Japan and in the West Indian black population (Blattner et al, 1982). The patients are virus and/or HTLV-antibody-positive and a few relatives to them have been shown to be HTLV-antibody-positive. These antibodies are directed against viral antigens secreted by the HTLV-positive cells (p19, p24), but the presence of antibodies on the surface of HTLV-infected T cells grown in vitro (detected by immunofluorescence) was also demonstrated testing HTLV-antibody-positive sera (Miyoshi et al, 1981). This indicates that leukemia-associated cell surface antigens might be a result of infection with oncogenic viruses in individuals susceptible to such infections.

BACKGROUND TO THE PRESENT STUDY

At the time of the start of our studies of humoral immunity in acute leukemia most studies reported to detect LAA in human acute leukemias had been performed in allogeneic systems. However, some studies of cell-mediated immune response and very few studies to detect humoral immune response to LAA had been performed in autochthonous systems. Some relevant studies of cell-mediated and humoral immune responses to LAA using different techniques will be presented here.

A. Cell-mediated immunity to acute leukemia

The cell-mediated immune response to leukemia-associated antigens has mainly been studied by mixed leukocyte culture tests (MLC) and T cell-mediated cytotoxicity in vitro and by cutaneous delayed hypersensitivity to leukemic cells or membrane preparations of them in vivo.

1. Mixed leukocyte culture tests

In these tests remission lymphocytes are mixed with autochthonous leukemic cells inactivated by irradiation or mitomycin C treatment. Reactivity in the MLC is thought to represent the presence of small lymphocytes capable of becoming sensitized to foreign antigens. If the cells are stimulated they will undergo blastogenic transformation and the following increased DNA synthesis is measured by the uptake of ^{3}H-thymidine. Early studies were performed by Fridman and Kourilsky (1969) and Viza et al (1969). In the latter study one patient was treated with stored irradiated autochthonous leukemic cells by subcutaneous injections. After 8 weeks of treatment a significant increased stimulation was observed in the MLC. A similar effect of autoimmunization was also noted by Powels et al (1971) in 4 patients with AML and 5 patients with ALL after immunotherapy with BCG and autochthonous leukemic cells in combination with chemotherapy. The capacity of the patients´ lymphocytes to respond to allogeneic cells was also increased but to a lesser extent, indicating a specific as well as a non-specific effect of the therapy.

Fefer et al (1974) performed MLC tests with cells from leukemic patients and their identical twins. Although lymphocytes from the leukemic patients were never stimulated by leukocytes from the normal twin, leukocytes from 3 patients, 1 AML and 2 ALL, stimulated the lymphocytes of their normal twins. The cells used in this study were not stored in liquid nitrogen before use, which often has been the case for stimulating cells in other studies. Gutterman et al (1973) performed studies of lymphocyte blastogenic response to autochthonous leukemic cells in 35 patients with acute leukemias. Leukemic cells were also analysed for the presence of membrane immunoglobulins in a direct immunofluroescence assay. A good clinical prognosis was positively correlated to a vigorous blastogenic response, its inhibition or facilitation by autochthonous serum and IgG bound to the leukemic cell surface. Only patients with AML showed positive membrane immunofluorescence on their own tumor cells. However, in this study it is not clear whether the positive membrane immunofluorescence on AML cells reflects IgG bound to the cells in the form of antibody to LAA or if it is due to binding to IgG Fc receptors. The MLC data suggested that patients with AML have a more intense immune response to their own leukemic cells than the patients with ALL (Gutterman et al, 1973).

2. Lymphocyte cytotoxicity

Rosenberg et al (1972) reported cytolytic effects on leukemic cells when exposed in vitro to lymphocytes of unrelated individuals or healthy family members of patients with acute leukemias. Cytotoxic lymphocytes were found in 46% of adult family members but only in 9% of children, which could reflect exposure to an environmental factor such as a virus. Freshly explanted leukemic cells of 2 patients with AML and 8 patients with ALL and cells from their identical twins were used as target cells. Lymphocytes of 20 individuals were cytotoxic to the leukemic cells only, in 8 cases the lymphocytes were reactive with the cells of both twins and 51 donors´ lymphocytes did not lyse cells from either twin. Never was reactivity observed against cells from the healthy twin only.

3. Cutaneous delayed hypersensitivity

Cutaneous delayed hypersensitivity to leukemic cells or membrane preparations of them has been reported by several authors (Oren and Herberman, 1971; Leventhal et al, 1972; Baker et al, 1974). In most studies positive reactions have been obtained during remission. In a combined study of 20 patients with AML and ALL 3 assays for cell-mediated immunity were used: MLC, lymphocyte cytotoxicity and cutaneous delayed hypersensitivity (Leventhal et al, 1972). All but one of the patients showed a positive response in at least one assay and it is to be observed that autochthonous material was used in all tests. While 4/8 leukemic patients were reactive with leukemic blast cell extracts in the skin test, all were negative with remission leukocyte extracts at the same protein concentration. Char et al (1973) reported cutaneous delayed hypersensitivity to membrane extracts of leukemic cells from patients with the same type of leukemia, but no reaction with extracts of remission cells or white blood cells from healthy donors. A more vigorous response was noted when the patients were tested in remission as compared to the acute stage of the disease.

B. Humoral immunity to acute leukemia

Earlier studies of antibodies against leukemic cells have been reviewed by Harris (1973) and in general these reports provided no convincing evidence for leukemic specificity. Antibody response to acute leukemia has been studied mainly by assays for complement-dependent cytotoxicity, antibody-dependent cellular cytotoxicity (ADCC), cytophilic antibodies and antibody binding assays including immunofluorescence and radioimmunoassays.

1. Complement-dependent cytotoxicity

Using a ^{51}Chromium release technique Dreesman et al (1973) demonstrated the presence of complement-dependent cytotoxic antibodies against autochthonous cells in 26 children with ALL. Sera of 10/12

patients contained high levels of cytotoxic antibodies against leukemic cells, while only 2/14 patients´ sera expressed similar levels of cytotoxicity against remission cells. Sera of 8/22 family members expressed cytotoxicity against leukemic cells,while none of the patients´ sera were cytotoxic to lymphocytes from healthy donors. Reactions to Ia antigens were not considered in the allogeneic combinations,neither the effect of chemotherapy on the specific antibodies. Bias et al (1972) and Doré et al (1976) also reported cytotoxic antibodies in sera of close relatives to patients with acute leukemia, but the specificity of such antibodies detected in allogeneic systems must be questioned in view of the great number of antigens shared by leukemic and normal blood cells. According to Mohanakumar and Raney (1978) antibodies reactive with Ia antigens explain many of the results claimed to demonstrate specificity for leukemia. Mann et el (1975) immunized patients with acute lymphoblastic leukemia in remission with BCG and Raji-cells and in some cases cytotoxic antibodies to autochthonous leukemic cells could be demonstrated. Before immunotherapy, however, no such antibodies could be shown.The antibody activity was lost before relapse or in prolonged remission despite continued Raji-cell administration. An immunosuppressive effect of chemotherapy was suggested as a possible explanation to this phenomenon. Metzgar et al (1975) were able to detect immunoglobulins on leukemic cells from patients with CML and CLL. After treatment of the cells with citrate buffer (pH 3.0),eluates from the cells were cytotoxic against leukemic cells from most patients with the same histological type of leukemia and were also cytotoxic against cells of 4/5 AML patients (CML-eluates), 4/9 AMML patients (CML-eluates) and 5/7 ALL patients (CLL-eluates). The specificity was similar to that described for non-human primate antisera (Metzgar et al, 1972) and the highest antibody titers were 1:4-1:8.

2. Antibody-dependent cellular cytotoxicity

Antibodies against allogeneic myeloblasts in sera of patients with AML were demonstrated by Hersey et al (1973) using human mononuclear blood cells as effector cells in an assay resembling antibody-dependent cellular cytotoxicity (ADCC). In patients treated with immunotherapy(consisting of BCG and allogeneic myeloblasts)in combination with chemotherapy a rise of antibody titers to high levels was noted. The activity suddenly fell after 3 months of therapy. Relapse was noted 2 weeks after this decrease of activity. Because of the allogeneic system no conclusion about the specificity of the reactions could be drawn.

3. Cytophilic antibodies

Antibodies cytophilic for macrophages and mediating their attachment to leukemic cells obtained from patients with AML and ALL were reported by Mitchell et al (1973). The antibodies were reactive with autochthonous leukemic cells in 7 patients with AML and 2 patients with ALL. In allogeneic systems they were only reactive with leukemic cells of the same histological type. Absorption with leukemic cells removed the cytophilic antibody in contrast to normal leukocytes and no reactivity was observed against remission cells from one patient with ALL. In 10/11 cases the antibody was of IgG class.

4. Antibodies detected by immunofluorescence

Using immunofluorescence techniques sera of some patients with acute leukemia have been shown to contain antibodies against a nuclear antigen (LANA) (Klein et al, 1974). Other sera contain antibodies binding to cell surface antigens (Baker et al, 1978). Antibodies to the nuclear antigen (LANA) were found in sera of 73% of patients with AML and considerably less frequently in sera of patients with other leukemias.

No such antibodies were detected in sera of healthy individuals. Gutterman et al (1973) investigating 35 patients with AML and ALL reported direct membrane immunofluorescence only on leukemic cells from patients with AML. The presence of immunoglobulins on the surface of the leukemic cells was related to a good clinical prognosis, but it was not shown whether the bound IgG reflected antibody binding to LAA or merely IgG bound to IgG Fc receptors on the cells. Cotropia et al (1977) also studied leukemic cells for the presence of membrane bound immunoglobulins in a direct immunofluorescence assay. 35 patients with AML and ALL were investigated and it was found that only cells from patients with AML had detectable amounts of immunoglobulins bound to the cell surface. The immunoglobulin was shown to be mainly of IgG class. Eluates after treatment of the leukemic cells with glycine buffer (pH 2.4) were shown to contain IgG molecules and Fab fragments, while eluates from normal leukocytes contained IgG molecules and Fc fragments. It was suggested that the presence of Fab fragments in eluates from the leukemic cells was due to IgG binding by the Fab part of the antibody, while Fc fragments in the eluates from normal leukocytes reflected binding via Fc to the Fc receptor. The expression of cell-associated proteolytic enzymes, as revealed by the presence of protease-modified anti-proteases, was postulated as an explanation of the cell surface IgG degradation. It was also postulated that the Fab binding to the leukemic cells might protect them from lysis by complement-dependent or antibody-dependent cellular cytotoxic mechanisms.

THE PRESENT INVESTIGATION

In order to study the humoral immune response to LAA in acute leukemias sera of patients were analysed for the presence of cytotoxic antibodies (papers I and II) as well as noncytotoxic antibodies (paper IV) to autochthonous leukemic target cells. Sera of patients with untreated AML were also investigated for the presence of specific immune complexes (paper III).
Target cells were prepared as illustrated in Figure 1.

Fig. 1

PREPARATION OF TARGET CELLS

Peripheral blood
or
Bone marrow aspirate

↓

Ficoll-Isopaque (1.077 g/cm^3)

↓

Interphase cells ← lymphocytes, monocytes, immature myloid cells

granulocytes
erythrocytes

↓

PBS with 20% fetal calf serum
and 10% DMSO

↓

Freezing

↓

Storage in liquid nitrogen (-196°C)

↓

Thawing

↓

^{51}Cr-labelling → Cytotoxicity assay

^{125}I protein A
bindning assay

I TUMOR-ASSOCIATED HUMORAL CYTOTOXICITY IN PATIENTS WITH ACUTE MYELOGENOUS LEUKEMIA BEFORE AND AFTER CHEMOTHERAPY

Methodological comments

A complement-dependent cytotoxicity assay using ^{51}Chromium-labelled target cells was developed in order to detect antibodies reactive with autochthonous leukemic target cells in sequential sera from patients with acute myelogenous leukemia. In contrast to earlier presented assays using ^{51}Chromium for labelling of target cells a very large amount of the isotope with high specific activity (200-500 Ci/g) was used. Usually 1 ml of ^{51}Chromium in the form of sodium chromate containing 1 mCi/ml was added to an equal amount of cell suspension. Only fresh lots of ^{51}Chromium were found to give adequate labelling. Exclusive use of ^{51}Chromium satisfying these specifications was found to be extremely important to get reliable results. Another important factor in the assay was the choice of complement donors. It was necessary to select a rabbit serum suitable as the source of complement for each patient studied to minimize interfering reactions due to heterophilic antibodies in rabbit sera. Incubation with complement for 15 min at room temperature (20-24^{o}C) was found to be optimal. Longer incubation times resulted in lower specific cytotoxic effects due to the heterophilic antibodies in rabbit sera.

Results

Cytotoxic antibodies against autochthonous leukemic cells were demonstrated in sequential sera of 8/8 patients with AML. No cytotoxic antibodies against autochthonous remission cells could be demonstrated in sera from 3 patients although the sera expressed significant cytotoxic activities against the patients' leukemic cells when tested in parallel. The cytotoxicity was increased after chemotherapy also in patients who did not enter the remission stage.

II DEMONSTRATION OF ANTIBODY-ASSOCIATED CELLULAR CYTOTOXICITY IN PATIENTS WITH ACUTE MYELOGENOUS LEUKEMIA BEFORE AND AFTER CHEMOTHERAPY

Methodological comments

Cytotoxic antibodies against autochthonous leukemic target cells were demonstrated in sequential sera obtained from patients with acute myelogenous leukemia using a newly developed ^{51}Chromium release assay called AACC (antibody-associated cellular cytotoxicity). The AACC reaction resembles ADCC (antibody-dependent cellular cytotoxicity) but three important points characterizing the AACC reaction should be noted: (1). The test system is completely autochthonous and there is no need for exogenous effector cells: (2). The AACC reaction occurs only in undiluted sera or at low serum dilutions: (3). The cytotoxicity appears very rapidly, being detectable already after incubation at room temperature (20-24^{o}C) for 15 min. While complement-dependent cytotoxicity of leukemic sera could be detected only under particular experimental conditions (See Paper 1), it was noted that another form of cytotoxic reaction could be detected in the absence of active complement. It was found that PBS with 1 mM $MgCl_2$, not containing $CaCl_2$ and addition of 10% heat-inactivated AB serum facilitated the AACC reaction and augmented the differences between control and leukemic sera. The incubation time with ^{51}Chromium was either 3h or 15-16h at 37^{o}C. The alternative giving optimal sensitivity in testing was chosen for each patient.

Results

Cytotoxic antibodies against autochthonous leukemic cells were demonstrated in 18/18 patients with AML. Sera of 4 patients expressing cytotoxicity against autochthonous leukemic cells showed no significant cytotoxic effects against autochthonous remission cells when tested in parallel. In most patients the

cytotoxicity was low in sera taken before therapy but increased after chemotherapy also in those patients in whom remission was not achieved.

The serum activity could be absorbed and eluted from a protein A-sepharose column and was recovered in the 7S-fraction after gel filtration on Sephadex G-200 and ion exchange chromatography, indicating that the antibodies involved are of IgG class.

III POSSIBLY SPECIFIC IMMUNE COMPLEXES IN SERA OF PATIENTS WITH UNTREATED ACUTE MYELOGENOUS LEUKEMIA

Methodological comments

Using a method described by Sjögren (1971) for dissociation of antigen-antibody complexes at low pH, the presence of complement-dependent cytotoxic antibodies was investigated in the retentates of ultrafiltrated sera obtained from patients with AML at the time of diagnosis. Amicon ultra filter membranes XM-100 (Amicon Corporation, Lexington, Mass., USA) were used, allowing molecules with MW less than 100 000 to pass through the membrane. The retentates containing molecules with MW more than 100 000, including all immunoglobulin classes, were tested for the presence of antibodies cytotoxic to autochthonous leukemic target cells in our complement-dependent cytotoxicity assay. The incubation time with ^{51}Chromium was either 3h at 37^{o}C or 15-17h at 37^{o}C. The alternative giving optimal sensitivity in testing was chosen for each patient.

Results

After ultrafiltration at low pH cytotoxic antibodies were detected in sera from patients with untreated AML when tested in our complement-dependent cytotoxicity assay. No specific antibodies could be detected in the sera before ultrafiltration. After ultrafiltration the retentates of all of 7 autochthonous and 3 of 4 allogeneic sera expressed significant cytotoxicity against leukemic target cells.

IV A ^{125}I PROTEIN A BINDING ASSAY DETECTING CELL SURFACE ANTIGENS: EVIDENCE FOR THE PRESENCE OF SPECIFIC ANTIBODIES AGAINST LEUKEMIA-ASSOCIATED ANTIGENS IN HUMAN LEUKEMIAS

Methodological comments

A ^{125}I protein A binding assay detecting antibodies to cell surface antigens on human blood and bone marrow cells was developed using sera from multitransfused nonleukemic patients sensitized against HLA antigens. The Bolton-Hunter reagent was used for iodination with ^{125}I according to the method described by Langone et al (1977). This reagent reacts spontaneously with the ε-amino groups of lysine under very mild conditions and the risk for over-iodination destroying protein A activity is low as compared to other methods introduced for iodination of this protein(using lactoperoxidase or chloramine T).PBS containing 3% bovine serum albumin (BSA) and 0.1% Tween-20 was used for washing and dilutions throughout the tests. Only freshly prepared solutions were used in the experiments to get reliable results. It was found important to bring down background radioactivity to low levels to increase the sensitivity of the assay. Every batch of ^{125}I protein A was therefore titrated against a panel of target cells in order to find the amount of ^{125}I protein A giving optimal sensitivity of the assay. Serum concentrations of IgG, IgA and IgM were determined by the technique of Laurell (1972).

Results

Antibodies binding to autochthonous leukemic cells were detected in sequential sera from 7 patients with AML and 2 patients with ALL. In 4 cases sera with demonstrable binding to the patients´ leukemic cells showed no significant binding to their own remission cells when tested in parallel. In all but 2 cases sera of patients in complete or partial remission showed the largest binding. It was found that differences in concentrations of total IgG, IgA and IgM in sera could not explain the differences in binding pattern of leukemic sera as compared to control sera.

GENERAL DISCUSSION

Most studies of humoral immune response to antigens associated with acute leukemias (LAA) have been performed with allogeneic combinations of cells and sera. Because of the possibility of immune reactivity against HLA-antigens, Ia antigens and some blood group antigens present on leukemic cells and on normal nucleated blood and bone marrow cells as well, studies of immune reactions in allogeneic systems claimed to be specific for LAA must be met with some scepticism. However, a few earlier studies in autochthonous systems indicated the presence of specific reactions against LAA in acute leukemia (See "background to the present study", B). These studies of humoral response to LAA and reports on cell-mediated immune reactions supporting the existence of specific LAA in acute leukemias inspired us to initiate analysis of humoral immune reactions against autochthonous leukemic cells primarily in acute myelogenous leukemia (AML). In the following discussion the interest will be focused on:

1. Antibodies to LAA detected by complement-dependent cytotoxicity
2. Antibodies to LAA detected by AACC (antibody-associated cellular cytotoxicity)
3. Antibodies to LAA detected by ^{125}I protein A binding assays
4. Circulating immune complexes in acute leukemia
5. Clinical utilization of antibodies to LAA
6. The nature of LAA

1. Antibodies to LAA detected by complement-dependent cytotoxicity

In our early studies of antibodies to LAA in autochthonous systems we used the ^{51}Chromium release technique and introduced a complement-dependent cytotoxicity assay sensitive enough to detect cytotoxic antibodies in sequential sera of 8/8 patients with AML (Paper 1). We also studied the effect of chemotherapy on the appearance of such antibodies. At the time of diagnosis most patients lacked detectable serum antibodies or showed very weak antibody activity to autochthonous leukemic cells. After chemotherapy cytotoxic antibodies became detectable in sera of all patients including those in whom remission was not achieved.

The appearance of antibodies after chemotherapy may be explained in at least four different ways: First, by reducing tumor burden, chemotherapy diminishes or eliminates the absorbing capacity of leukemic cells and thereby leaves an excess of specific circulating antibodies detectable in the complement-dependent cytotoxicity assay. Second, it is known from other malignant diseases that tumors may produce soluble antigens with the same specificity as those on the cell membranes. Thus, reduced shedding of soluble leukemia antigen as a consequence of the reduced tumor mass might result in diminished formation of circulating antigen-antibody complexes. This could also result in higher amounts of free circulating leukemia antibodies. These explanations anticipate that the antibody-forming cells are not seriously affected by the chemotherapy. Third, antigen-antibody complexes are anticomplementary and theoretically could interfere with endogenous complement factors, thereby blocking lysis of leukemic cells induced by antibody and complement. By addition of sera from 28/39 AML patients in the acute stage of the disease Mann et al. (1974) noted varying degrees of blocking of the cytotoxic effects of rabbit heteroantisera on the leukemic cells. Fourth, the possibility of a decreased suppressor T lymphocyte function as a result of the chemotherapy should also be mentioned. This might result in an increased antibody production leading to the appearance of cytotoxic antibodies in patients' sera.

Sera of 3/3 AML patients failed to express significant cytotoxicity against autochthonous remission cells, although the sera were cytotoxic against autochthonous leukemic cells when tested in parallel (Paper I). This indicates that the antibodies are leukemia-specific and that cells expressing LAA are not present in the peripheral blood during remission or are greatly reduced in number. Lack of reactivity against autochthonous remission cells among sera which are reactive with autochthonous leukemic cells has been confirmed with other techniques in later studies (Paper II; Paper IV; Bertini et al., 1982).

In acute lymphoblastic leukemia (ALL) a few reports on the presence of cytotoxic antibodies reactive with autochthonous leukemic cells in complement-dependent cytotoxicity assays have been published. Using a ^{51}Chromium release technique high levels of cytotoxic antibodies reactive with autochthonous leukemic cells from 10/12 children

with ALL were demonstrated by Dreesman et al. (1973). Only 2/14 patients' sera were equally cytotoxic to autochthonous remission cells. Mann et al. (1975) treated ALL patients in remission with immunotherapy consisting of BCG and Raji-cells (a tissue culture B lymphocyte cell line) in combination with chemotherapy. Cytotoxic antibodies against autochthonous leukemic cells could not be demonstrated after chemotherapy alone, but in a few cases such antibodies appeared after addition of immunotherapy. We were, however, able to demonstrate cytotoxic antibodies against autochthonous AML leukemic cells after chemotherapy alone (See above). Loss of antibody activity in sequential sera despite continued Raji-cell administration was suggested to be due to the immunosuppressive effect of the chemotherapy (Mann et al., 1975).

2. Antibodies to LAA detected by AACC

In our later studies of antibodies to leukemic cell surface antigens in AML a new ^{51}Chromium release assay independent of active complement and resembling antibody-dependent cellular cytotoxicity (ADCC) was developed (Paper 11). Because the demonstrated activity could be distinguished from ADCC in some important respects, we preferred to call it antibody-associated cellular cytotoxicity (AACC).We believe that this reaction is mediated via Fc receptor bearing cells in the same way as ADCC. However, there is no need for exogenous effector cells as in ADCC and thus AACC is completely autochthonous. The effector cells have not yet been identified and characterized, but passage through a nylon wool column or incubation in plastic Petri dishes does not reduce the cytotoxic reaction. This form of cytotoxicity is dependent on a specific serum antibody and also the presence of autochthonous target and effector cells (present in the band of mononuclear blood cells after Ficoll-Isopaque separation). Furthermore, AACC differs from ADCC in two important respects: (a) it occurs only with undiluted sera or at very low serum dilutions, (b) the reaction is very rapid, being detectable already after incubation for 15 min at room temperature.

The results obtained with the AACC assay were similar to those obtained with the complement-dependent cytotoxicity assay. Cytotoxic antibodies could be demonstrated in 18/18 AML patients

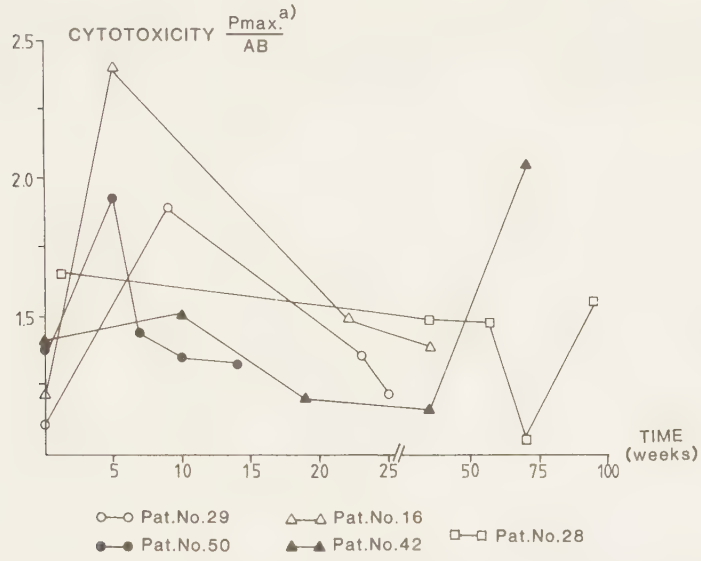
reacting with autochthonous leukemic target cells. AACC activities were low or absent in most sera obtained at the time of diagnosis, but appeared in sequential sera after chemotherapy also in those patients in whom remission was not achieved. A peak level of cytotoxicity was often found after some weeks of chemotherapy, followed by a decline until the patients relapsed. After chemotherapy in the relapse stage elevated cytotoxicity could be demonstrated again in the sera of two patients as shown in Figure 2.

In order to compare AACC activities between patients, cytotoxicity ratios were calculated: (a) The ratio between the cytotoxicity of each patient's serum showing the highest level of cytotoxicity (P_{max}) and control serum (AB) was higher in patients who entered remission as compared to those in whom remission was not achieved, $p<0.0005$. This is illustrated in Figure 3. (B) Similar results were obtained by calculation of the ratio between the cytotoxicity of each patient's maximally cytotoxic serum (P_{max}) and the serum obtained at diagnosis (PA). Remission patients showed higher cytotoxic activities in sera as compared to patients who did not enter the remission stage, $p<0.01$. This is illustrated in Figure 4. The results might indicate that patients with a strong humoral immune response to LAA have a better clinical prognosis than patients with a weak response (See below, 5). Another explanation might be that the total mass of leukemia antigen left in patients after chemotherapy is lower in remission patients, thereby leaving free antibodies in detectable concentrations (See above, 1).

Remission cells obtained from 4 patients with AML were tested with autochthonous sera in the AACC assay. Sera containing cytotoxic antibodies against autochthonous leukemic cells expressed no significant cytotoxicity against the patients' remission cells when tested in parallel. This finding provides strong evidence for LAA specificity and shows that leukemia antigen bearing cells are not present in the peripheral blood during remission or are greatly reduced in number (See above, 1).

Variation of cytotoxicity against autochthonous leukemic cells in five patients with AML assayed in sera harvested at different time points of the disease.

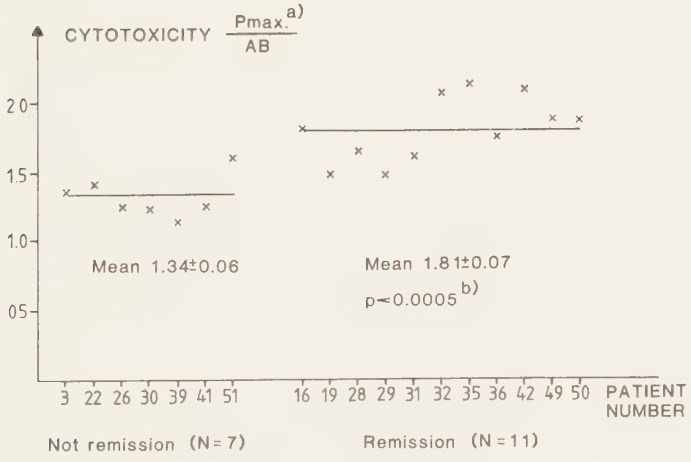
Fig.2



a) The ratio between the activities of each patient's maximally cytotoxic serum (P_{max}) and control serum (AB).

Comparison of cytotoxicity between the group of patients which entered remission (N = 7) and the group in which remission was not achieved (N = 11).

Fig.3

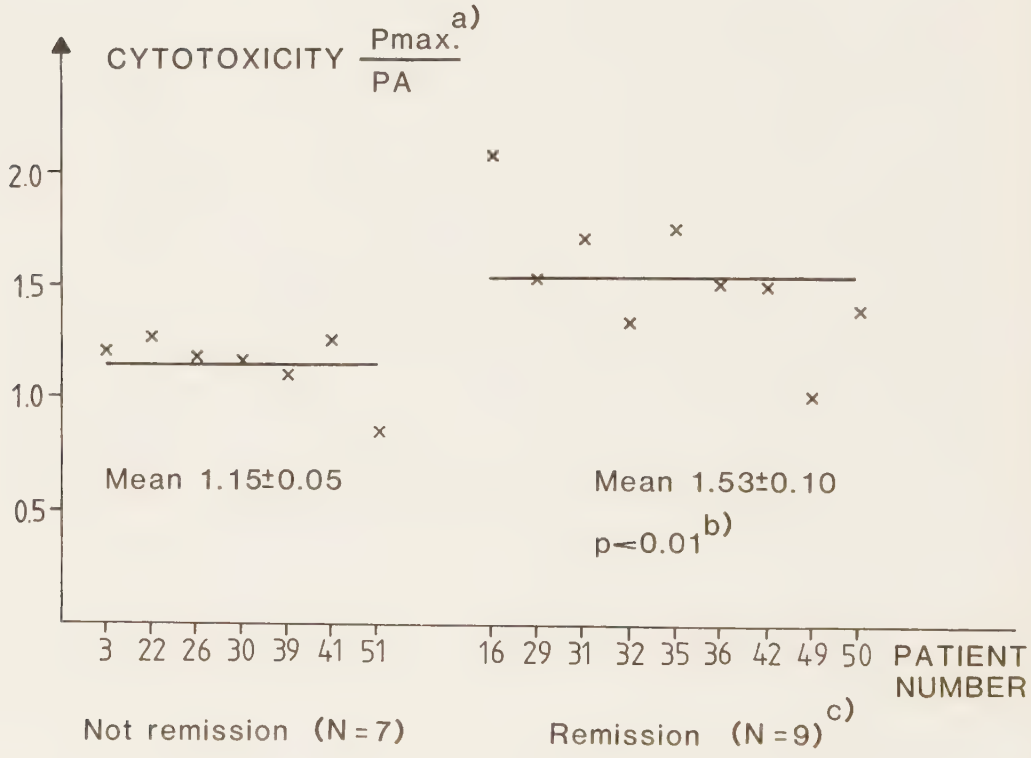


a) The ratio between the activities of each patient's maximally cotytoxic serum (P_{max}) and control serum (AB).

b) The statistical significance of the difference between the groups of patients was calculated according to Student's t-test.

Comparison of cytotoxicity between the group of patients which entered remission (N = 7) and the group in which remission was not achieved (N = 9).

Fig.4



a) The ratio between the activities of each patient's maximally cytotoxic serum (P_{max}) and the serum obtained at diagnosis.

b) The statistical significance of the difference between the groups of patients was calculated according to Student's t-test.

c) Serum PA-28 and remission serum from pat. no. 19 were not available for testing.

3. Antibodies to LAA detected by ^{125}I protein A binding assays

A ^{125}I protein A binding assay was developed to study also non-cytotoxic antibodies in patients with AML and ALL (Paper IV). Antibodies capable of binding to autochthonous leukemic cells were demonstrated in 7 patients with AML and 2 patients with ALL successfully treated by chemotherapy. The results were shown to be similar to those obtained with the cytotoxic techniques (See above, 1 and 2). No significant binding was registered to autochthonous remission cells (4 patients studied) and in most cases the antibodies increased after chemotherapy. It was also concluded that variations in the concentrations of total immunoglobulins could not explain the differences in binding capacities of patients´sera as compared to control sera. Similar results in AML have very recently been presented by Bertini et al. (1982). In this study antibodies capable of binding to autochthonous leukemic cells were detected using a ^{125}I protein A binding assay with target cells fixed in glutaraldehyde. No such antibodies could be demonstrated in patients with ALL (4 patients studied). We were, however, able to demonstrate antibodies binding to autochthonous leukemic cells in ALL (2 patients studied) (Paper IV). After chemotherapy the antibodies were constantly detectable or appeared in those patients who were negative at the time of diagnosis (Bertini et al., 1982). The specificity of the reactions was checked by testing autochthonous remission cells. As in our studies no significant binding was registered to the patients´ own remission cells.

4. Circulating immune complexes (CIC) in acute leukemia

A method for dissociation of antigen-antibody complexes in sera and separation of their components by ultrafiltration at low pH has been described by Sjögren et al. (1971). In patients with untreated AML we used this technique to detect potentially cytotoxic antibodies against autochthonous leukemic cells (Paper III). Antibodies could be detected in sera in all of 7 autochthonous and 3 of 4 allogeneic combinations. Thus, indicating that in sera of patients in the acute stage of the disease potentially cytotoxic antibodies are bound to soluble LAA in the form of circulating antigen-antibody complexes. Alternate explanations are: (a) By competition at the antigen site interfering non-cytotoxic antibodies prevent the

cytotoxic antibodies to express cytotoxicity. (b) Anti-idiothypic antibodies bind to the Fab part of the cytotoxic antibodies, thereby preventing them to bind to the leukemic cells. If interfering non-cytotoxic antibodies or anti-idiothypic antibodies are more sensitive to acid treatment, they may be destroyed, leaving more acid resistant cytotoxic antibodies free to express cytotoxicity. However, the present knowledge about the sensitivity of immunoglobulins to pH 3.1 makes these explanations less probable.

The presence of CIC in sera of patients with acute leukemias has been demonstrated in several studies (Carpentier et al., 1977; Claque et al., 1978; Mod et al., 1980; Carpentier et al., 1981; Bertini et al., 1982). At the time of diagnosis AML patients (40-50%) have elevated levels of CIC, while the frequency is lower in ALL. In patients without CIC the remission rate has been shown to be significantly higher (as compared to patients with detectable CIC) (Carpentier et al., 1981; See part two, "circulating immune complexes"). The clinical usefulness of CIC measurements was also demonstrated in some patients with AML demonstrating a rise in CIC up to 3 months before clinical relapse (Carpentier et al., 1981). In these studies the composition of the CIC was not investigated. The possibility that the antigen part of CIC is composed of bacterial antigens in AML patients was not supported by the findings reported by Carpentier et al. (1981), which demonstrated lack of correlation between the presence of CIC and evident sepsis.

The biological significance of CIC in tumor patients has been a matter of debate. In human acute leukemia CIC probably interfere with complement-dependent cytotoxicity (Mann et al., 1974; Paper III) and ADCC-like reactions (Hersey et al., 1973). Furthermore, in larger amounts CIC may block cell-mediated tumor immunity (Hellström et al., 1971).

5. Clinical utilization of antibodies to LAA

In the clinical situation antibodies to LAA may be used for (a) monitoring the patients and (b) immunotherapy.

(a) In our study of cytotoxic antibodies in AML we were able to separate 2 groups of patients. Remission patients showed higher cytotoxic activities when tested in the AACC assay as compared to patients who did not enter the remission stage (See above, 2). Thus, monitoring the humoral response to LAA in AML patients may be of value in evaluation of the prognosis. In AML (21/26 patients) Baker et al. (1979) reported increased immune reactivity of bone marrow cells for 1 to 6 months (mean 3.7) before relapse diagnosed by standard morphological criteria using mouse heteroantisera reactive with AML leukemic cells. Use of reinduction therapy at this point may prolong remission duration and survival.

(b) In AML patients subjected to immunotherapy (with BCG and allogeneic myeloblasts) in combination with chemotherapy, assays for complement-dependent cytotoxicity and indirect immunofluorescence showed antibodies to a panel of allogeneic AML cells in 10/16 patients (Baker et al., 1978). The specificity of the antibodies was checked by testing remission cells from AML patients and leukemic cells from patients with CML, ALL and CLL. Reactivity was significantly higher against allogeneic AML cells as compared to allogeneic remission cells and leukemic cells from the other types of leukemia. Furthermore, specificity was indicated by absorption analysis. It was also found that patients with high antibody response survived longer and had longer remission duration than patients with low levels of antibodies ($p<0.05$).

Immunotherapy (consisting of BCG and allogeneic AML cells or a leukemic antigen preparation) in combination with chemotherapy resulted in an antibody response in 2/8 AML patients treated with whole leukemic cells and in 5/13 patients treated with the antigen (Granatek et al., 1981). Duration of remission was 159 weeks for antibody responders and 75 weeks for nonresponders and length of survival was 164 weeks vs. 98 weeks.

In vitro immunotherapy utilizing monoclonal antibody against LAA has been used with encouraging results (Ritz et al., 1981). Residual leukemic cells are assumed to be present in remission bone marrow. Monoclonal antibody reactive with cALL leukemic cells and addition

of rabbit complement have been used to lyse such residual leukemic cells in this form of acute leukemia. After such in vitro treatment remission bone marrow has been used to "rescue" the patients following high dose chemoradiotherapy.

In vivo treatment with monoclonal antibodies to LAA has been used with partially successful results in patients with T-cell leukemia and lymphomas (For review, see Ritz and Schlossman, 1982). In addition, monoclonal antibodies can be used as carriers of other cytotoxic agents (such as ricin toxin or chemotherapy drugs) in order to reduce tumor load (Ghose et al., 1978).

6. The nature of LAA

The present investigation of humoral immune response to LAA as well as a few other studies of immune reactivity to autochthonous leukemic cells (See "background to the present study", B) support the existence of specific antibodies reactive with molecules linked to the leukemic cell surface. It should be realized that, theoretically, LAA may be formed as a result of virus infection or even genetic mutation as well as rearrangements secondary to exposure to chemicals, radiation etc. Qualitatively new antigens have been demonstrated in one form of T-cell leukemia, where virus-related antigens have been shown to be associated with the leukemic cells (For review, see Gallo and Wong-Staal, 1982). In some close relatives to the leukemic patients an antibody response to these antigens has also been demonstrated.

Concluding remarks

It is concluded from our data and reports by others that antibodies recognizing LAA often appear in acute leukemia patients. There are indications that these antibodies may be clinically relevant in terms of monitoring response to therapy, including various immunological manipulations (See above, 5). Before the clinical practicability may be adequately ascertained, these findings need further confirmation in patients subjected to CIC eliminating treatment, e.g. plasmapheresis, to treatment with immunostimulants or to immunization with

antigens. Furthermore, it should be of great interest to verify e.g. the significance of the appearance of immunoglobulins (including various subclasses) not only as free antibodies to LAA, but also as a part of CIC in patients with acute leukemias (in progress).

MAIN FINDINGS IN THE PRESENT STUDY

1. Cytotoxic antibodies against autochthonous leukemic target cells have been detected in sequential sera of 8/8 patients with AML using a complement-dependent cytotoxicity assay. In most patients no such antibodies were detected at the time of diagnosis but appeared after chemotherapy also in those patients who did not enter clinical remission. They were reactive with leukemic cells but unreactive with remission cells from the same patients when tested in parallel (Paper I).

2. Cytotoxic antibodies against autochthonous leukemic target cells have been detected in sequential sera of 18/18 patients with AML using an assay resembling antibody-dependent cellular cytotoxicity, which we have designated AACC (antibody-associated cellular cytotoxicity). This assay does not require addition of exogenous effector cells in contrast to ADCC (antibody-dependent cellular cytotoxicity. The reaction is very fast and is most pronounced in undiluted sera. Results analogous to those of the complement-dependent cytotoxicity assay were obtained with respect to rise of activity after chemotherapy and lack of reactivity with remission cells (Paper II).

3. Potentially cytotoxic antibodies against autochthonous leukemic target cells were detected in sera of untreated patients with AML using a method know to split antigen-antibody complexes by ultra-filtration at low pH in combination with the complement-dependent cytotoxicity assay. After such treatment cytotoxic antibodies were detected in all of 7 autochthonous and 3 of 4 allogeneic sera investigated (Paper III).

4. $A^{125}I$ protein A binding assay was established to detect also noncytotoxic antibodies in sequential sera of patients with acute leukemias. By this assay antibodies binding to autochthonous leukemic target cells were demonstrated in 7 patients with AML and 2 patients with ALL successfully treated by chemotherapy. No significant binding was registered to autochthonous remission cells and in most cases the antibodies increased after chemotherapy. It was also shown that the

increased binding of patients' sera as compared to control sera was not just a reflection of increased immunoglobulin concentrations (Paper IV).

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PART TWO - HEAT PRODUCTION INDUCED BY THE INTERACTION OF IMMUNE COMPLEXES WITH NORMAL BLOOD CELLS

INTRODUCTION

Development of modern calorimeters has made it possible to measure very small heat effects, such as a few μW (microwatt). Since all biochemical processes are accompanied by heat evolution or heat absorption, it follows that calorimetric methods are nonspecific, which may limit the usefulness of the reactions. However, lack of specificity can also be advantageous, allowing measurements of total cellular metabolic activity in normal as well as pathological conditions. Monitoring total cellular heat production may also uncover phenomena which could be obscured using a specific assay.

Heat production in resting cells reflects the basal metabolic rate of the cells. In blood cells with a capacity for phagocytosis most of the glucose is then catabolized anaerobically. During phagocytosis, however, glucose metabolism is mainly aerobic through the hexose monophosphate shunt (HMPS), resulting in an increased oxygen consumption. An increased oxygen consumption is also seen in granulocytes after exposing them to preformed antigen-antibody complexes, anti-neutrophil antibodies and some other agents active on the cell membrane. Great interest has been focused on the participation of circulating immune complexes (CIC) in the pathogenesis of several diseases, including not only a variety of autoimmune and infectious diseases but also several types of neoplastic diseases. Hitherto most studies of immune complexes (IC) in these conditions have been focused on whether IC are present or not. In our studies calorimetry has been used to study a biological effect of preformed antigen-antibody complexes on various populations of human blood cells. The purpose of the study was to follow the effect on heat production in blood by addition of various amounts of IC and also to try to delineate to which cell type any increase of heat production could be ascribed.

BACKGROUND TO THE PRESENT STUDY

1. Circulating immune complexes

Formation of circulating immune complexes (CIC) in vivo may be considered as a physiological mechanism to eliminate soluble antigens. Normally CIC are rapidly cleared from the blood through the reticuloendothelial system. Several other structures have been shown also to clear CIC. One example is the mesangium of the glomerulus, which appears to be the primary defense of the kidney against CIC (Kunkel, 1980). Elevated CIC levels have been observed in some healthy individuals and have been shown to vary significantly when measured repeatedly during a period of 24 hours (Di Mario et al, 1981). Increased levels have also been found in normal pregnancy (Stimson et al, 1981). However, highly elevated levels of CIC are thought to play a significant role in the pathogenesis of several diseases (WHO, 1977). CIC are usually found in sera of patients with a variety of autoimmune and infectious diseases (Barnett et al, 1979; Williams et al, 1981; Ritzman and Daniels, 1982) as well as in patients with several types of neoplasms, including for instance neuroblastoma (José and Seshadri, 1974), melanoma (Rossen et al, 1978), lung cancer (Füst et al, 1981) and acute leukemia (Carpentier et al, 1977; Claque et al, 1978; Mod et al, 1980; Carpentier et al, 1981; Bertini et al, 1982). The antigen part of the complex has been shown to vary with regard to size, valence, number of specificities and physicochemical properties.

Assays for detection of CIC are principally based either on the presence of immunoglobulins or of complement components such as C1q and C3b. Beacuse of variations of the antibodies in CIC with regard to immunoglobulin class, subclass, affinity, avidity and complement-binding properties on one side and the amount of complement components on the other, CIC are very heterogenous. Furthermore, the composition varies with the type of disease and during the course of the disease in the same individual. These facts explain why the results of methods developed for detection of CIC correlate poorly with each other and with disease activity (Füst et al. 1981). In SLE, however , the solid phase C1q binding assay (SC1q)

has been found to be a reliable indicator of disease activity, while the fluid phase Clq binding assay (FClq), anti-DNA antibody titer and C3 concentrations were neither associated with nor predictive of clinical activity of the disease (Abrass et al, 1980).

In acute leukemia it has been reported that appearance of CIC correlates with progression of the disease. The remission rate has also been reported to be significantly higher in patients without CIC as compared to those with detectable complexes, 79 percent vs 26 percent (Carpentier et al, 1981).

2. Interaction of immune complexes with blood cells

Several types of human blood cells are capable to bind CIC through receptors for not only the Fc portion of the immunoglobulin molecule, but also for C3b, a cleavage product of C3. Fc receptors for IgG molecules are present on human granulocytes (Henson, 1969; Messner and Jelinek, 1970; Ishizaka et al, 1970; Hofmeister et al, 1981), monocytes (LoBuglio et al, 1967; Huber et al, 1968; Ishizaka et al, 1970), B lymphocytes (Dickler and Kunkel, 1972; Theofilopoulos et al, 1974) and T lymphocyte suppressor cells (Moretta et al, 1977). Fc receptors for IgM molecules are found on T helper lymphocytes (Moretta et al, 1977), while Fc receptors for IgA molecules are present on a subpopulation of human B lymphocytes (Gupta et al, 1979). The IgE antibodies, i e the reagins, bind to IgE receptors on basophilic cells (Ishizaka et al, 1970).

Receptors for C3b are found on granulocytes (Henson, 1969; Eden et al, 1973; Hofmeister et al, 1981), monocytes (Huber et al, 1968) and B lymphocytes (Bianco et al, 1970; Theofilopoulos et al, 1974). The C3b receptors are responsible for the immune adherence phenomenon. Human platelets are immune adherence negative and react with antigen-antibody complexes through their Fc-receptors (Pfueller and Lüscher, 1972).

3. Metabolic activation induced by immune complexes

It was reported already in 1960 that the addition of antigen antibody complexes increases the oxygen uptake by human blood in vitro (Strauss and Stetson). Rossi et al (1970) then demonstrated that antileukocyte antibodies as well as antigen-antibody complexes induce a dramatic increase of the oxygen uptake and a marked increase of glucose oxidation through the hexose monophosphate shunt (HMPS). Similar increases in oxygen consumption and HMPS activity also occur in granulocytes within a few seconds after addition of particles and prior to significant particle ingestion (Goldstein and Weissman, 1979). Metabolic activation is initiated by signals starting in the cell membrane when granulocytes are exposed to particles or immune complexes and independent of phagocytosis. Starkebaum et al (1981) reported that soluble as well as insoluble immune complexes stimulated granulocytes to enhanced glucose oxidation via the HMPS, although insoluble immune complexes were most effective.

Analysis with a flow calorimeter has shown that heat production increases in leukocytes during phagocytosis of certain bacteria or polystyrene latex particles (Levin, 1973a). In systemic lupus erythematosus (SLE) Levin and Thomasson (1974) found increased leukocyte heat production when cells suspended in plasma were exposed to homogenates prepared from autochthonous white blood cells. It was suggested that stimulation of leukocyte metabolism and the resulting heat production was due to the presence of antigen-antibody complexes, although this could not be proven. In other calorimetric studies a marked increase of heat production was observed when aerobic metabolism via the HMPS was stimulated in both erythrocytes (Levin, 1973b; Monti and Wadsö, 1976) and leukocytes (Levin, 1973b).

THE PRESENT INVESTIGATION

Our intention was to develop an assay based on calorimetry in order to study the cellular events following the interaction of preformed immune complexes with human blood cells. The results of these investigations are reported in papers V and VI and are summarized as follows:

V A NEW APPROACH TO DETECTION OF ANTIGEN-ANTIBODY COMPLEXES BY MICROCALORIMETRIC MEASUREMENTS OF HEAT PRODUCTION IN BLOOD CELLS

Methodological comments

The experiments were performed with LKB-10700-2 batch microcalorimeters. The calorimetric vessel has two compartments containing blood cells and preformed immune complexes (IC), respectively, in these experiments. Rotation of the calorimetric vessel causes immediate mixing of cells and IC and the process is started. The heat produced by cellular activation is transformed into electrical energy by a thermopile surrounding the vessel. The electrical signal generated is directly proportional to heat production. A major advantage characterizing this type of calorimeter is that rapid reactions can be monitored already from the beginning of the experiments. It should be noted that activation and control experiments were conducted simultaneously by use of twin calorimeters. Anyhow, the colorimeters must be equilibrated for about 2h before the process is started.

Results

Marked heat production was found when whole venous blood was mixed *in vitro* with preformed antigen-antibody complexes. It was found that the quantitative proportions between antigens (ovalbumin and human serum albumin) and their specific antibodies were important for the rate of heat production with a maximum reached at the zone of equivalence.

V1 HEAT PRODUCTION IN DIFFERENT POPULATIONS OF HUMAN BLOOD CELLS EXPOSED TO IMMUNE COMPLEXES IN VITRO: THE IMPORTANCE OF THE FC PARTS OF IMMUNOGLOBULINS AND THE INFLUENCE OF ACTIVE COMPLEMENT

The experiments were performed with the same type of calorimeter as in paper V.

Results

Granulocytes could be shown to be responsible for the main part of the heat production recorded in whole venous blood after the addition of preformed immune complexes in vitro. Mononuclear cells before and after removal of monocytes, i.e. lymphocytes,were also activated. Platelets and purified erythrocytes were on the other side not activated. Immune complexes (IC) consisting of antigen and $F(ab)_2$ fragments were shown to initiate heat production only in the presence of active complement, while IC consisting of antigen and intact antibodies activated the cells in the absence of active complement as well. Small but constantly higher activation values were found when granulocytes or washed blood cells were preincubated in heat inactivated serum as compared to active serum before mixing with the IC.

GENERAL DISCUSSION

Circulating immune complexes (CIC) are assumed to play a significant role in the pathogenesis of several diseases (WHO, 1977). CIC are very heterogenous with variations in their composition as to antigen, antibody, proportion between antigen and antibody and the presence of complement components (C1q, C3b). A number of assays has been used to identify CIC in attempts to correlate their presence with various diseases and/or the activity of the disease. However, the methods have often given discordant results, correlating poorly with each other and with disease activity (Füst et al., 1980). Most of the previous methods established for the study of IC have been limited to recognize their presence. Our intention was to develop an assay to study a biological effect of preformed IC on various populations of human blood cells.

We used calorimetry as a tool for such studies. A marked increase in heat production was found when whole venous blood was mixed with preformed IC _in vitro_ (Paper V). Maximal heat production was recorded when antigens and antibodies were mixed at a proportion, reflecting the so called zone of equivalence. Heat production in different populations of blood cells interacting with IC was also investigated (Paper VI), and it was found that granulocytes were responsible for most of the heat production in whole blood after admixture of IC. Unfractionated mononuclear cells could also be activated by IC and the heat produced was approximately 25 percent of that produced in a corresponding number of granulocytes. Monocyte-depleted mononuclear cells, i.e. nonphagocytizing lymphocytes, could still be activated by the complexes.

When platelets were exposed to IC no increased heat production was recorded. This was unexpected since it has been reported that platelets are metabolically stimulated by such complexes (Mustard and Packham, 1968). If binding of IC to the Fc receptor on platelets is a prerequisite for activation of heat production, a sterical block through complement fixation to the Fc region of IgG may by an explanation for lack of heat production as suggested by Pfueller and Lüscher (1972).

In human granulocytes interaction of IC with receptors on the cell membrane causes increased glucose oxidation (through the hexose monophosphate shunt, HMPS) (Goldstein and Weissman, 1979). This metabolic activation leads to secondary phenomena, e.g. increased oxygen consumtion (Strauss and Stetson, 1960), production of toxic oxygen radicals accompanied by chemiluminescence (Starkebaum et al., 1981) and heat production (Paper VI). The binding of IC to granulocytes and subsequent activation of the cells are mediated by Fc and C3b receptors on the cell surface (Klebanoff and Clark, 1980) and this is in agreement with our results (Paper VI). In the absence of active complement the heat production initiated by IC was probably induced through binding of IC to the Fc receptor since IC consisting of antigen and $F(ab)_2$ fragments prepared from the specific antibodies did not stimulate the cells (Paper VI). In the presence of active complement such complexes were able to initiate heat production in the cells, probably due to complement activation through

the alternative pathway and subsequent C3b generation. This finding is supported by the data of Gadd and Reid (1980) which showed that $F(ab)_2$ fragments of IgG antibodies bound to antigen are able to generate C3b through the alternative pathway of complement activation.

In the absence of active complement a small but constantly higher heat production was recorded when granulocytes or unseparated blood cells were reacting with IC (as compared to reactions where active complement was present)(Paper VI). This was somewhat unexpected, since in the presence of active complement, it has been reported a considerably higher degree of attachment as well as ingestion of IC by rabbit granulocytes (Mantovani, 1975). However, our cell preincubation with serum for 2 h before mixing with the complexes may explain this difference in heat production. In other experiments we have noted an increased heat production in granulocytes mixed with IC in the presence of active complement as compared to reactions where it was absent (unpublished observations). These divergent results may be explained through the effect of cell preincubation with serum, which was not performed in these experiments. Complement-dependent inhibition of binding of IC to cell membranes after preincubation of the cells in fresh serum has been reported by Erdei et al. (1980) and can be an explanation to our findings (Paper VI). A complex effect of complement on the interaction between IC and cell membranes, which under various specified conditions resulted in either increased or decreased binding, has been reported by Miller and Nussenzweig (1974).

In conclusion, our present experience reveal that calorimetric methods are suitable for monitoring heat production in blood cells interacting with IC formed *in vitro*. Such studies would allow identification of pathological cells in situations where it is difficult to discriminate between normal and pathological cells by use of conventional methods. In ongoing studies we are investigating the interaction of preformed IC with populations of blood cells obtained from patients with acute leukemia.

Preliminary results in AML patients in remission indicate differences in basal heat production in granulocytes as compared to healthy individuals. Furthermore, after exposure to IC, heat production is less than normal, which may indicate defective Fc and/or C3b receptors on granulocytes from AML patients in remission. These results are in line with observations by Hofmeister et al. (1981) in acute leukemia, which show defective C3b receptor on morphologically normal granulocytes obtained from the patients when the disease was progressing. In remission, however, C3b receptor function was found to be normal. Furthermore, using calorimetry for studies on the interaction between immune complexes and cells, it might be possible to evaluate not only the effect of variations in the composition of IC (with regard to antigen, antibody and complement components) but also the significance of e.g. complement and complement activation in the regulation of such interactions (in progress).

MAIN FINDINGS IN THE PRESENT STUDY

1. An assay has been established to detect preformed antigen-antibody complexes based on heat production in human blood cells when mixed with solutions containing such complexes (Paper V).

2. Increase of heat production in blood cells to which preformed antigen-antibody complexes had been added was dependent on the proportions between antigens and their specific antibodies. Maximal activation of the blood cells was obtained with antigens and antibodies mixed at proportions reflecting the so called zone of equivalence (Paper V).

3. Granulocytes are responsible for the main heat production when whole blood is mixed with preformed antigen-antibody complexes (Paper VI).

4. Mononuclear blood cells before and after elimination of phagocytic cells show increased heat production when mixed with preformed antigen-antibody complexes, indicating that also lymphocytes are activated by such complexes (Paper VI).

5. Immune complexes consisting of antigen and $F(ab)_2$ fragments prepared from the specific antibodies are able to initiate heat production in granulocytes only in the presence of active complement (Paper VI).

6. Small but constantly higher activation values were found when unseparated blood cells or granulocytes were preincubated in heat inactivated serum as compared to active serum before mixing with immune complexes (Paper VI).

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TUMOR-ASSOCIATED HUMORAL CYTOTOXICITY IN PATIENTS WITH ACUTE MYELOGENOUS LEUKEMIA BEFORE AND AFTER CHEMOTHERAPY

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Sera of eight unselected adult patients with acute myelogenous leukemia obtained before and after chemotherapy were repeatedly tested for specific complement-dependent cytotoxicity against autochthonous peripheral white blood cells from the acute leukemia stage and from the remission stage, respectively. Complement-dependent cytotoxicity against white blood cells from the acute leukemia stage was demonstrated in all of the eight patients, while none of three patients' sera were reactive against white blood cells from the remission stage tested in parallel. The cytotoxicity was increased after chemotherapy, also in those patients in whom remission was not achieved.

Evidence has been accumulated for the existence of tumor-specific antigens associated with human acute leukemia cells (Harris *et al.*, 1971; Metzgar *et al.*, 1972) and various immune responses against these antigens have been demonstrated by complement fixation, immunofluorescence, immune adherence and cytotoxicity tests (Dore *et al.*, 1967; Halterman *et al.*, 1972; Gutterman *et al.*, 1973; Klein *et al.*, 1974). Cytophilic antibodies were demonstrated by Mitchell *et al.* (1973) and antibody-dependent cellular cytotoxicity was described by Hersey *et al.* (1973). Cellular immunity was demonstrated *in vivo* by delayed hypersensitivity skin tests (Oren and Herberman, 1971; Leventhal *et al.*, 1972) and *in vitro* by the mixed lymphocyte culture test (Viza *et al.*, 1969; Powles *et al.*, 1971; Leventhal *et al.*, 1972; Gutterman *et al.*, 1973) and cellular cytotoxicity assays (Leventhal *et al.*, 1972, Rosenberg *et al.*, 1972). Only a few attempts have been made to study alterations of these immune responses in the course of the disease and as a result of therapy.

Patients with acute myelogenous leukemia are usually offered chemotherapy with various combinations of cytostatic drugs with or without corticosteroids. In this way it is possible to achieve remission in 50-65% of adult patients and the median duration of survival from onset of first remission has been reported to be 295 days (Fairley, 1976). As an adjunct to chemotherapy, immunotherapy in the form of specific and non-specific stimulation of the immune response against leukemia-associated antigens has been reported to be beneficial in patients with acute leukemias. BCG immunotherapy as well as immunization with irradiated or neuraminidase-treated allogeneic leukemia cells has been shown to prolong remission periods and median survival of patients with acute lymphoblastic (Mathé *et al.*, 1969) and myelogenous leukemias (Freireich *et al.*, 1976; Holland *et al.*, 1976). Many clinical and experimental reports thus suggest that immunological defense mechanisms are activated in patients with acute leukemias and that they may be further stimulated by immunological manipulation. These immune reactions against leukemia antigens are likely to be influenced by treatment of the patients with cytostatic drugs.

To shed some light on the effect of chemotherapy on specific immune reactions against leukemia antigens, we studied specific humoral complement-dependent cytotoxicity in the present investigation. Sequential sera of eight patients with acute myelogenous leukemia were collected in the course of the disease and were tested in parallel against autochthonous peripheral white blood cells.

MATERIAL AND METHODS

Patients

Eight patients with acute myelogenous leukemia were studied in the present investigation. Five of them were treated at Lund University Hospital and three of them at Borås Community Hospital. Some important clinical and laboratory data are recorded in Table I. Six of the patients were given cyclic chemotherapy with different combinations of cytostatic drugs as specified in Table I and two were given daily treatment with an experimental drug, prednimustine, to which were added one and four injections of vincristine respectively. Peripheral white blood cells from the acute stage of the disease were obtained before any clinical treatment was begun and peripheral white blood cells from the remission stage of the disease were obtained when no chemotherapy had been given for the last 3 weeks. Sera from the patients were taken before therapy and then sequentially in the course of the disease in periods without drug treatment.

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TABLE I

CLINICAL AND LABORATORY DATA ON EIGHT INVESTIGATED PATIENTS WITH ACUTE MYELOGENOUS LEUKEMIA AT THE TIME OF SERUM COLLECTION

Pat. No., sex and age (years)	Serum [1]	Number of peripheral white blood cells $\times 10^{-3}$ per mm^3 and % of blast cells		% of blast cells in the bone marrow	Clinical stage of the disease	Chemotherapy [4]
20	PA-20	18.5	40%	Dry tap [3]	Before treatment	—
Male, 26	PB-20	3.6	ND [2]	18%	After 4 weeks of treatment	TA×1
	PC-20	1.7	3%	30%	After 6 weeks of treatment without remission	TA×2
22	PA-22	20.0	15%	56%	Before treatment	—
Female, 24	PG-22	12.0	ND	ND	After 19 weeks of treatment without remission	0×4+prednimustine daily
25	PA-25	6.5	4%	46%	Before treatment	—
Male, 43	PB-25	ND	ND	ND	After 2 weeks of treatment	Prednimustine daily
	PC-25	3.3	0%	ND	After 6 weeks of treatment without remission	0×1+prednimustine daily
29	PA-29	88.0	47%	83%	Before treatment	—
Male, 50	PF-29	2.0	0%	ND	After 10 weeks of treatment	TRAP×4
	PG-29	4.0	0%	<5%	Complete remission after 18 weeks of treatment	TRAP×4+COAP×4
32	PA-32	149.0	95%	97%	Before treatment	—
Male, 44	PB-32	3.8	6%	89%	After 6 weeks of treatment	TA×2
	PE-32	4.4	0%	<5%	Complete remission after 19 weeks of treatment	TA×2+TRA×1+POMP×1 +COA×1+TOMP×2
35	PA-35	49.0	15%	68%	Before treatment	—
Female, 36	PD-35	2.0	0%	<5%	Complete remission after 9 weeks of treatment	TRAP×4
36	PA-36	15.4	24%	Dry tap	Before treatment	—
Female, 31	PB-36	3.7	ND	10%	After 6 weeks of treatment	TA×2
	PC-36	2.0	0%	<5%	Complete remission after 12 weeks of treatment	TA×4
	PD-36	9.6	0%	<5%	Complete remission after 24 weeks of treatment	TA×4+TRA×1+POMP×1
	PE-36	9.8	0%	<5%	Complete remission after 30 weeks of treatment	TA×4+TRA×1+POMP×1 +TA×1
37	PA-37	6.4	7%	86%	Before treatment	—
Female, 32	PC-37	5.4	0%	<5%	Complete remission after 9 weeks of treatment	TRAP×4

[1] Sera designated PA: patients' sera taken before any treatment; PB—PG: patients' sera taken at various stages of the disease during chemotherapy. The letters are followed by the patient's number. — [2] Not done. — [3] Unsuccessful bone-marrow aspiration as a consequence of packed cells in bone marrow. — [4] The following abbreviations are used: TA = thioguanine-cytarabine, O = vincristine, Prednimustine = chlorambucil-prednisolone, TRAP = thioguanine-daunorubicin-cytarabine-prednisolone, COAP = cyclophosphamide-vincristine-cytarabine-prednisolone, TRA = thioguanine-daunorubicin-cytarabine, POMP = mercaptopurine-vincristine-methothrexate-prednisolone, COA = cyclophosphamide-vincristine-cytarabine, TOMP = thioguanine-vincristine-methothrexate-prednisolone.

Sera

Normal sera from healthy AB blood group donors and sera from patients with acute myelogenous leukemia collected at the acute stage of the disease before therapy and in the course of the disease were stored at −20° C. They were inactivated at 56° C for 30 min immediately before use.

Complement

Rabbit sera in dilutions of 1/4-1/20 were used as the source of complement. Before testing of each patient, several complement batches were screened and a suitable rabbit was selected as complement donor. Complement batches with significant cytotoxic activity against respective target cells were excluded. Only a few sera were found to be suitable as the source of complement for target cells from a given donor. Complement was inactivated by heating to 56° C for 30 min.

Estimation of immunoglobulins

The concentration of immunoglobulin classes IgA, IgG and IgM in sera was determined by the technique of Laurell (1972).

Preparation of target cells

Peripheral white blood cells were separated from heparinized whole venous blood by layering onto Ficoll-Isopaque (specific gravity 1.077) and centrifugation for 10 min at 70 *g* followed by 15 min at 700 *g*. The cells in the interphase were collected and after washing were suspended in phosphate-buffered saline (PBS) with 20% fetal calf serum and 10% DMSO. The cells were frozen by cooling at the rate of 1° C/min to −60° C and then stored in liquid nitrogen. After thawing in a 40° C water bath, cell viability was 85-95% and recovery was 70-90%, with no difference observed between normal white blood cells and white blood cells from the acute or remission stages of patients with acute myelogenous leukemia.

In vitro *assay of complement-dependent cytotoxicity*

Peripheral white blood cells from patients with acute myelogenous leukemia in the acute stage of the disease before therapy and remission cells from three of the patients harvested within 4 months of onset of remission were used as target cells after storage in liquid nitrogen. Peripheral white blood cells from healthy blood donors were stored and used in the control experiments.

After thawing in a 40° C water bath the cell suspensions were washed in PBS with 10% fetal calf serum and 2% Hepes at 37° C, then $3\text{-}5\times10^6$ viable cells were incubated for 3-6 h in 1 ml PBS with 5% fetal calf serum, 1% Hepes and 0.5 mCi/ml of ^{51}Chromium in the form of sodium chromate (New England Nuclear, Boston, Mass., USA, spec. act. 200-500 Ci/g). Only fresh lots of ^{51}chromium solution were used in the experiments. After the incubation the fluid was removed and 1.0 ml of fetal calf serum containing 2 drops (0.05 ml) Varidase (Lederle Laboratories, Wayne, New Jersey, USA) (10 000 IU/ml) was added to the cells. After approximately 30 sec, 10 ml PBS with 10% fetal calf serum and 2% Hepes and 0.7 ml Varidase were added and the cell suspension was left at 4° C for 20-25 h.

Before testing, the fluid was removed and replaced by 10 ml PBS with 10% fetal calf serum and 2% Hepes, then the cell suspension was incubated for 30-60 min at 4° C. Aliquots of 1.0 ml cell suspension containing $1.0\text{-}1.5\times10^5$ viable cells/ml were centrifugated at 400 *g* for 2 min and the sedimented cells were resuspended in test serum of the desired dilution. All experiments were performed in duplicate or triplicate sets. A final concentration of 2.5×10^4 cells/0.1 ml of test serum was incubated for 15 min at room temperature. During the incubation the tubes were occasionally shaken. Next, 0.5 ml of rabbit complement diluted in PBS with 10% fetal calf serum and 2% Hepes was added and the cell suspensions were incubated for another 15 min at room temperature. After addition of 4 ml PBS with 10% fetal calf serum and 2% Hepes cooled to 4° C the cells were centrifuged at 700 *g* for 10 min, and after 45 min at 4° C 1 ml of the supernatant and the original tubes were counted in a gamma counter. The percentage of the isotope release was calculated as follows:

$$\frac{\text{Radioactivity in 1.0 ml of supernatant}\times100\times4.6}{\text{Total radioactivity/tube}}$$

The statistical significance of the recorded differences between complement-dependent effects of test and control sera was calculated by Student's t-test.

RESULTS

Eight patients with acute myelogenous leukemia were studied and the results are summarized in Tables I-VI and Figure 1. Data reflecting some important laboratory and clinical parameters of the disease at the time of serum collection are

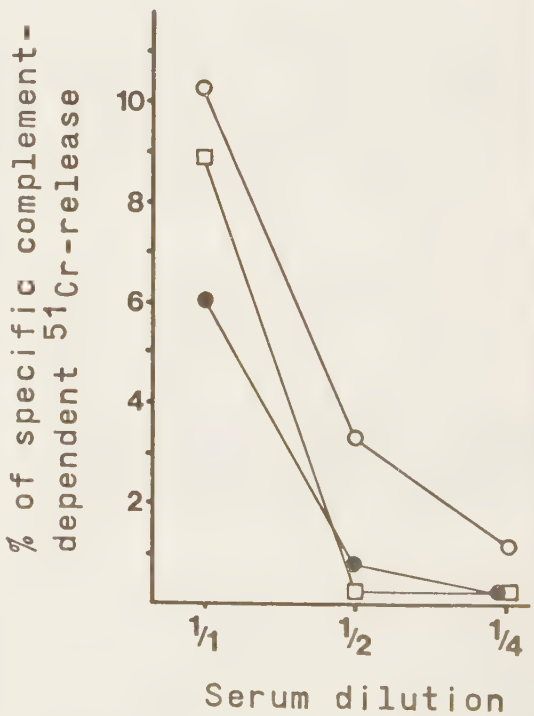

FIGURE 1

Titration by the ^{51}Cr-release technique of the specific cytotoxic activity of three sera from patients with acute leukemia. Specific complement-dependent ^{51}Cr-release means the difference in complement-dependent cytotoxicity between patients' sera and normal sera of the corresponding dilution.

presented in Table I. Sera taken before treatment at the acute stage of the disease and sera collected after chemotherapy were tested for complement-dependent cytotoxicity against autochthonous peripheral white blood cells and their activities were compared to those of sera from healthy AB blood group donors. The target cells harvested in the acute stage of the disease were stored in liquid nitrogen until they were thawed immediately before the test was performed. Specific complement-dependent cytotoxicity was demonstrated in 8/8 patients with acute myelogenous leukemia (Table II). The cytotoxic activity of patients' sera before chemotherapy was usually low or statistically not significant, but increased in the course of treatment with cytostatic drugs to statistically highly signi-

TABLE II

COMPLEMENT-DEPENDENT CYTOTOXICITY OF AUTOCHTHONOUS SERA AGAINST WHITE BLOOD CELLS OF PATIENTS WITH ACUTE MYELOGENOUS LEUKEMIA AT DIFFERENT STAGES OF THE DISEASE AS COMPARED TO SERA OF HEALTHY BLOOD DONORS

Experiment	Serum [1]	Complement [2]		^{51}Cr-release ±SE	% complement-dependent ^{51}Cr-release	Difference to [3] control serum	Clinical stage of the disease
1, Pat. No. 25	AB-7	KC-63 1:20	+	8.4±0.3	0.2 \	—	
			−	8.2±0.3	1.1		
	AB-8	KC-63 1:20	+	9.6±0.3	2.0 /	—	
			−	7.6±0.5			
	PA-25	KC-63 1:20	+	6.5±0.8	2.1	1.0 $p>0.05$	Before treatment
			−	4.4±0.7			
	PB-25	KC-63 1:20	+	7.4±0.1	2.8	1.7 $p<0.05$	After 2 weeks of treatment
			−	4.6±0.5			
	PC-25	KC-63 1:20	+	12.3±0.8	6.7	5.6 $p<0.01$	After 6 weeks of treatment without remission
			−	5.6±0.2			
2, Pat. No. 20	AB-7	KC-50 1:5	+	10.9±0.4	−0.1 \	—	
			−	11.0±0.9	−0.2		
	AB-9	KC-50 1:5	+	9.8±0.1	−0.3 /	—	
			−	10.1±0.2			
	PA-20	KC-50 1:5	+	27.0±0.1	13.1	13.3 $p<0.0005$	Before treatment
			−	13.9±0.4			
	PB-20	KC-50 1:5	+	26.5±0.7	16.9	17.1 $p<0.0005$	After 4 weeks of treatment
			−	9.6±0.5			
	PC-20	KC-50 1:5	+	31.1±0.5	19.3	19.5 $p<0.0005$	After 6 weeks of treatment without remission
			−	11.8±0.3			
3, Pat. No. 32	AB-10	KC-82 1:4	+	21.8±1.6	−0.8 \	—	
			−	22.6±0.6	−0.3		
	AB-12	KC-82 1:4	+	23.9±0.1	0.3 /	—	
			−	23.6±1.6			
	PA-32	KC-82 1:4	+	36.6±0.2	0.4	0.7 $p>0.05$	Before treatment
			−	36.2±0.6			
	PB-32	KC-82 1:4	+	32.5±2.9	7.5	7.8 $p<0.05$	After 6 weeks of treatment
			−	25.0±0.3			
	PE-32	KC-82 1:4	+	35.4±2.1	15.1	15.4 $p<0.01$	Complete remission
			−	20.3±1.5			
4, Pat. No. 37	AB-7	KC-3 1:4	+	16.1±0.2	1.8	—	
			−	14.3±0.7			
	PA-37	KC-3 1:4	+	17.4±0.3	2.4	0.6 $p>0.05$	Before treatment
			−	15.0±0.1			
	PC-37	KC-3 1:4	+	24.5±0.8	12.1	10.3 $p<0.01$	Complete remission
			−	12.4±0.1			

(Continued on next page)

TABLE II *(continued)*

Experiment	Serum [1]	Complement [2]		^{51}Cr-release ±SE	% complement-dependent ^{51}Cr-release	Difference to [3] control serum	Clinical stage of the disease
5, Pat. No. 35	AB-10	KC-3 1:4	+	15.4±1.3	2.4	—	
			−	13.0±0.8			
	PA-35	KC-3 1:4	+	20.7±0.5	7.3	4.9 $p<0.05$	Before treatment
			−	13.4±0.1			
	PD-35	KC-3 1:4	+	21.8±0.1	9.7	7.3 $p<0.01$	Complete remission
			−	12.1±0.2			
6, Pat. No. 29	AB-11	KC-83 1:4	+	21.2±1.1	2.2	—	
			−	19.0±0.4			
	PA-29	KC-83 1:4	+	26.0±1.0	5.3	3.1 $p>0.05$	Before treatment
			−	20.7±0.6			
	PF-29	KC-83 1:4	+	25.6±1.8	5.3	3.1 $p>0.05$	After 10 weeks of treatment
			−	20.3±0.4			
	PG-29	KC-83 1:4	+	28.6±1.1	8.1	5.9 $p<0.05$	Complete remission
			−	20.5±0.6			
7, Pat. No. 22	AB-10	KC-3 1:4	+	15.1±0.0	2.6	—	
			−	12.5±0.2			
	PA-22	KC-3 1:4	+	19.1±0.5	7.9	5.3 $p<0.01$	Before treatment
			−	11.2±0.3			
	PG-22	KC-3 1:4	+	22.1±1.3	11.3	8.7 $p<0.01$	After 19 weeks of treatment without remission
			−	10.8±0.4			
8, Pat. No. 36	AB-15	KC-80 1:4	+	10.0±0.7	−1.0	—	
			−	11.0±0.0			
	PA-36	KC-80 1:4	+	12.7±0.2	0.6	1.6 $p<0.05$	Before treatment
			−	12.1±0.4			
	PB-36	KC-80 1:4	+	12.5±0.9	−0.5	0.5 $p>0.05$	After 2 weeks of treatment
			−	13.0±1.2			
	PC-36	KC-80 1:4	+	17.1±0.4	6.0	7.0 $p<0.01$	Complete remission
			−	11.1±0.5			
	PD-36	KC-80 1:4	+	14.5±0.1	2.9	3.9 $p<0.01$	Complete remission
			−	11.6±0.1			
	PE-36	KC-80 1:4	+	15.2±1.1	4.2	5.2 $p<0.01$	Complete remission
			−	11.0±0.2			

[1] Sera designated AB: sera of healthy donors of blood group AB followed by the donor's number; PA: patients' sera taken before treatment with cytostatics. PB—PG as patients' sera taken at various stages of the disease. The latters are followed by the patients' number. — [2] Rabbit sera KC-3—KC-83 were used as unheated (+) and heated to 56° C for 30 min (−), respectively, and diluted to the denoted dilution. — [3] The probability that the recorded difference is due to chance was calculated by Student's t-test.

ficant levels independent of the type of chemotherapy and clinical stage of the disease. No correlation was found between the activities of patients' sera and the percentage of myeloblasts in the peripheral blood. Even in three patients without remission, elevated complement-dependent cytotoxicity was registered after a period of intensive chemotherapy. The cytotoxic activity was most pronounced in undiluted sera, in some of the sera it was still demonstrable at a dilution of 1/2 and it decreased to insignificant levels at serum dilutions 1/4 (Fig. 1).

The specificity of the cytotoxicity was further checked by parallel testing of sequential sera from three patients with acute myelogenous leukemia for complement-dependent cytotoxicity against autochthonous peripheral white blood cells obtained at both the acute and the remission stages of the disease (Table III). Sera expressing statistically highly significant effects on autochthonous white blood cells harvested during the acute stage of the disease showed no complement-dependent cytotoxic effects on white blood cells obtained from the same patients at the remission stage of the disease. The

TABLE III

COMPLEMENT-DEPENDENT CYTOTOXICITY OF AUTOCHTHONOUS SERA AGAINST WHITE BLOOD CELLS OBTAINED AT ACUTE AND REMISSION STAGES OF ACUTE MYELOGENOUS LEUKEMIA

Experiment	Serum [1]	Complement [2]		Target cells: White blood cells from the acute stage of the disease [3] % ^{51}Cr-release ± SE	% complement-dependent ^{51}Cr-release	Difference to control serum [3]	White blood cells from the same patient during remission [3] % ^{51}Cr-release ± SE	% complement-dependent ^{51}Cr-release	Difference to control serum [3]
1, Pat. No. 36	AB-13	KC-80 1:4	+	15.4±0.7	0.9	—	30.5±0.9	1.9	—
			−	14.5±0.3			28.6±0.2		
	PA-36	KC-80 1:4	+	16.1±0.2	1.2	0.3 $p>0.05$	34.8±0.3	1.6	−0.3 $p>0.05$
			−	14.9±0.4			33.2±0.2		
	PC-36	KC-80 1:4	+	21.5±1.8	7.4	6.5 $p<0.05$	30.7±1.4	0.8	−1.1 $p>0.05$
			−	14.1±0.2			29.9±0.6		
2, Pat. No. 35	AB-10	KC-3 1:8	+	6.5±0.5	1.5	—	8.6±0.4	1.2	—
			−	5.0±0.2			7.4±0.8		
	PA-35	KC-3 1:8	+	6.4±0.1	1.9	0.4 $p>0.05$	8.1±0.8	−2.2	−3.4 $p<0.05$
			−	4.5±0.2			10.3±0.4		
	PD-35	KC-3 1:8	+	12.3±0.8	4.7	3.2 $p<0.05$	10.8±0.1	0.6	−0.6 $p>0.05$
			−	7.6±0.1			10.2±0.3		
3, Pat. No. 37	AB-13	KC-3 1:4	+	10.9±0.3	1.0	—	17.5±1.1	−0.2	—
			−	9.9±0.3			17.7±0.3		
	PA-37	KC-3 1:4	+	15.5±0.7	5.9	4.9 $p<0.01$	21.2±0.4	2.3	2.5 $p>0.05$
			−	9.6±0.2			18.9±0.3		
	PC-37	KC-3 1:4	+	21.8±3.4	12.4	11.4 $p<0.05$	18.7±0.2	0.9	1.1 $p>0.05$
			−	9.4±0.8			17.8±1.5		

[1] See footnote [1] to Table II. — [2] See footnote [2] to Table II. — [3] See footnote [3] to Table II.

experimental conditions were identical when white blood cells from the acute and remission stages were tested and both target cells were tested simultaneoulsy.

Since the non-specific complement-dependent cytotoxicity with sera from healthy AB blood group donors was most pronounced on white blood cells obtained during remission, these cells seem to be more sensitive in general to complement-mediated cytotoxicity than white blood cells taken at the acute stage of the disease. This suggests that the reason for the lack of effect of the patients' sera on these cells was the absence of the appropriate leukemia-associated antigens rather than a general insensitivity to cytotoxicity mediated by complement.

When sera from patients with acute myelogenous leukemia taken before therapy were tested for cytotoxicity against peripheral white blood cells from healthy donors (Table IV) no significant cytotoxic activity was registered as compared to autochthonous and AB blood group control sera.

When an adequate complement donor had been found for each patient, sera of the leukemia patients were repeatedly tested. Highly reproducible results were obtained with sera showing high cytotoxic activity as PC-20, as well as sera showing lower levels of cytotoxicity as PD-35 (Table V). However, with serum PC-25, expressing a low cytotoxic activity, statistically significant effects were demonstrable only in 3/7 tests. Serum PC-37 repeatedly showed high cytotoxic activity during 3.5 months, but after that time the cytotoxic activity was completely lost despite identical test conditions. Apparently the cytotoxic activity of this serum had been lost during storage at −20° C.

Reference control AB-sera never showed any reproducible complement-dependent cytotoxicity against white blood cells from the acute stage of myelogenous leukemia. Three to five reference control AB-sera were tested repeatedly against peripheral white blood cells of every leukemia patient.

To establish whether the differences in cytotoxic activities of patients' sera before and after chemotherapy are due to variation of total immunoglobulins as a consequence of the disease and its

TABLE IV

TESTS FOR COMPLEMENT-DEPENDENT CYTOTOXICITY OF SERA FROM SIX PATIENTS WITH ACUTE MYELOGENOUS LEUKEMIA ON LYMPHOCYTES OF HEALTHY DONORS AS COMPARED TO AUTOCHTHONOUS SERA

Experiment	Serum [1]	Complement [2]		% ^{51}Cr-release ± SE	% Complement-dependent ^{51}Cr-release	Difference to [3] control serum
1, Lymphocyte donor H.S.	H.S.	KC-81 1:4	+	34.6±1.5	1.0	—
			−	33.6±1.6		
	AB-13	KC-81 1:4	+	33.3±0.3	1.1	0.1 p>0.05
			−	32.2±1.3		
	PA-20	KC-81 1:4	+	32.1±0.9	−1.1	−2.1 p>0.05
			−	33.2±0.4		
	PA-25	KC-81 1:4	+	39.1±2.0	3.9	2.9 p>0.05
			−	35.2±1.2		
	PA-36	KC-81 1:4	+	33.5±0.2	0.6	−0.4 p>0.05
			−	32.9±1.6		
2, Lymphocyte donor AB-14	AB-14	KC-2 1:4	+	17.0±0.9	0.3	—
			−	16.7±0.0		
	PA-20	KC-2 1:4	+	18.6±0.5	−1.5	−1.8 p>0.05
			−	20.1±0.5		
	PA-25	KC-2 1:4	+	24.7±1.4	1.7	1.4 p>0.05
			−	23.0±0.1		
	PA-37	KC-2 1:4	+	23.2±0.2	−0.1	−0.4 p>0.05
			−	23.3±1.6		
3, Lymphocyte donor AJ	AJ	KC-83 1:4	+	16.9±0.3	1.3	—
			−	15.6±1.4		
	AB-12	KC-83 1:4	+	11.1±0.3	1.7	0.4 p>0.05
			−	9.4±0.6		
	AB-13	KC-83 1:4	+	11.5±0.5	1.3	0.0 p>0.05
			−	10.2±0.9		
	PA-25	KC-83 1:4	+	14.3±0.2	0.0	−1.3 p<0.05
			−	14.3±0.1		
	PA-32	KC-83 1:4	+	14.3±0.4	1.7	0.4 p>0.05
			−	12.6±0.0		
	PA-35	KC-83 1:4	+	14.6±0.1	1.9	0.6 p>0.05
			−	12.7±1.5		

[1] See footnote 2 to Table I. H.S. and A.J. are autochthonous sera from the respective lymphocyte donors. — [2] Rabbit sera KC-2 KC-83 were used as complements unheated (+) and heated to 56° C for 30 min (−), respectively, and diluted to the denoted dilution. — [3] See foot note [3] to Table II.

treatment, the concentrations of IgA, IgG and IgM in some of the tested sera were estimated (Table VI). There was no parallelism between the specific cytotoxic activities and amounts of total IgA, IgG or IgM immunoglobulins.

DISCUSSION

Several studies have confirmed the presence of leukemia-associated antigens in man (Harris *et al.*, 1971; Metzgar *et al.*, 1972; Halterman *et al.*, 1972) and antibody response to leukemia cells from patients with acute myelogenous leukemia has been demonstrated with various techniques. Immunofluorescence studies have shown positive membrane fluorescence with anti-IgG on peripheral white blood cells from patients with acute myelogenous leukemia (Gutterman *et al.*, 1973). With the anti-complement immunofluorescence test a positive staining was found in the nuclei (Klein *et al.*, 1974). Cytophilic antibodies (Mitchell *et al.*, 1973) and antibody-dependent lymphocyte killing of acute myelogenous leukemia cells has also been demonstrated (Hersey *et al.*, 1973).

Halterman *et al.* (1972) demonstrated complement-dependent cytotoxicity against leukemia cells from

TABLE V

REPRODUCIBILITY OF COMPLEMENT-DEPENDENT CYTOTOXICITY DETECTABLE WITH EIGHT PATIENTS' SERA REPEATEDLY TESTED AGAINST AUTOCHTHONOUS PERIPHERAL WHITE BLOOD CELLS FROM THE ACUTE STAGE OF MYELOGENOUS LEUKEMIA

Serum [1]	Frequency of tests demonstrating [2] significant specific complement-dependent cytotoxicity	Average % specific complement-dependent cytotoxicity ± SE	Number of reference control AB-sera	Time period [2] (months)
PC-20	6/6	11.8±1.9	3	1.5
PG-22	5/5	9.6±2.6	4	4
PC-25	3/7	3.0±0.8	2	0.5
PG-29	8/10	3.9±0.5	5	7.5
PE-32	3/3	10.2±2.8	3	1.5
PD-35	8/10	3.7±0.7	3	4
PC-36	6/8	3.5±0.8	3	2.5
PC-37	6/10 [3]	5.6±1.6	4	5

[1] See footnote [1] to Table II. — [2] The patients' sera were tested repeatedly during the indicated time period. The significance of serum activities in comparison to reference control AB-sera was judged by Student's t-test. — [3] Serum PC-37 lost all cytotoxic activity after 3.5 months of storage at −20° C. All six tests performed prior to this time point showed significant cytotoxicity (average 9.3±0.9).

TABLE VI

LEVELS OF IMMUNOGLOBULINS IN SERA OF THREE PATIENTS WITH ACUTE MYELOGENOUS LEUKEMIA AND TWO HEALTHY DONORS

Serum [1]	IgG [2]	IgA [2]	IgM [2]
PA-35	14.5	2.2	1.3
PD-35	12.4	1.7	1.1
PA-36	7.8	0.8	0.8
PC-36	14.5	0.9	0.5
PA-37	8.8	1.3	0.5
PC-37	8.4	1.2	0.4
AB-10	8.2	1.2	0.4
AB-13	8.5	1.3	0.9

[1] See footnote [1] to Table II. — [2] The values are expressed as g/l. Normal values: IgG: 6.5-15, IgA: 0.75-2.4, IgM: 0.3-2.0.

patients with acute myelogenous leukemia with heterologous antisera. However, in this case, sera from rabbits immunized with a papain-soluble cell membrane component from a tissue-culture cell line of the Burkitt lymphoma Raji were used. These antisera reacted with peripheral white blood cells from patients with both acute myelogenous leukemia and acute lymphoblastic leukemia but showed no reactivity against peripheral white blood cells from healthy individuals or against remission cells from patients with leukemia. In patients with acute leukemia the reactivity of these antisera was correlated to the percentage of immature cells in the bone marrow and not to the percentage of immature cells in the peripheral blood. The same authors later demonstrated complement-dependent cytotoxicity against autochthonous leukemic peripheral white blood cells in sera of 3/4 patients with acute myelogenous leukemia in remission after immunization with the Raji lymphoid tissue-culture cell lines, whereas they were unable to demonstrate specific complement-mediated cytotoxicity before the immunization or after chemotherapy alone (Mann *et al.*, 1975).

The present investigation differs from the above-mentioned reports in three essential ways. (1) Specific complement-dependent humoral cytotoxicity in patients with acute myelogenous leukemia was studied in autochthonous systems using sequential sera from the patient before and after chemotherapy. (2) Specific complement-dependent humoral cytotoxicity was demonstrated in all (8/8) of the patients with acute myelogenous leukemia. (3) Specific complement-dependent humoral cytotoxicity was found to increase after chemotherapy.

The cytotoxicity was generally low or statistically not significant before treatment but it appeared in some cases already after a very short time of chemotherapy and showed the highest activity in the remission stage. Patients No. 20, 22 and 25 showed significant cytotoxic activity despite the lack of complete remission.

However, all three patients were given intensive chemotherapy during the last 2 weeks before serum collection. No correlation was found between the cytotoxic activities in sera and the percentage of immature cells in the peripheral blood.

The appearance of specific complement-dependent cytotoxicity in sera of patients with acute myelogenous leukemia after chemotherapy may be explained in at least two ways. By reducing the tumor burden, chemotherapy diminishes or eliminates the absorbing capacity of leukemia cells and thereby leaves an excess of specific circulating antibodies. Under such conditions they can be more easily detected in a complement-mediated cytotoxicity test. Alternatively, reduced shedding of soluble leukemia antigen as a consequence of the reduced tumor mass might result in diminished formation of antigen/antibody complexes. This could also result in higher amounts of free circulating leukemia antibodies. This explanation anticipates that the antibody-forming cells are not seriously affected by the chemotherapy. By quantitating immunoglobulins in sera of patients with acute myelogenous leukemia before and during chemotherapy we could eliminate the possibility that differences in the specific cytotoxicity were due to major changes in total immunoglobulin concentrations.

When autochthonous remission white blood cells taken within 4 months of onset of remission were used as target cells and tested in parallel with cells obtained at the acute stage of the disease, no cytotoxic activity could be demonstrated in 3/3 patients' sera that were active on the latter cells. This indicates that the cytotoxic activity of the sera is leukemia-specific and that the leukemia-antigen-bearing cells are not present in the peripheral blood during remission or are greatly reduced in number. This is in accordance with the findings of Halterman *et al.* (1972) and Mann *et al.* (1975).

Since all the patients received blood transfusions at an early stage of the disease, sequential sera from the patients were not tested against allogeneic peripheral white blood cells because of the difficulty in distinguishing anti-leukemic antibodies from induced anti-HL-A and other blood group antibodies. Further support for the specificity of the cytotoxicity to leukemic cells was obtained by testing patients' sera obtained before therapy on allogeneic white blood cells from healthy donors. No cytotoxic activity was found in patients' sera as compared to autochthonous sera and allogeneic sera from healthy AB blood group donors. On the other hand, some of the test sera taken before therapy were significantly cytotoxic to allogeneic peripheral white blood cells from patients with acute myelogenous leukemia as compared to allogeneic sera from healthy blood donors (Fäldt and Ankerst, unpublished observations).

The demonstrated increase in specific complement-dependent humoral cytotoxicity following chemotherapy is analogous to the observation in rats with polyoma-virus-induced sarcomas in which cytotoxic antibodies could not be detected in sera obtained before treatment with a cytostatic drug, but became demonstrable during remission after cyclophosphamide treatment (Steele *et al.*, 1974).

The establishment of correlations between alterations in levels of specific antibody cytotoxicity and the clinical course of the leukemia might lead us to the use of tests for such antibodies in the evaluation of chemotherapy and as a guide for comparison of various therapeutic procedures. They are of obvious interest in providing an additional *in vitro* parameter for evaluation of immunotherapeutic procedures.

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CYTOTOXICITÉ HUMORALE ASSOCIÉE A LA TUMEUR DANS DES CAS DE LEUCÉMIE MYÉLOGÈNE AIGUË, AVANT ET APRÈS LA CHIMIOTHÉRAPIE

Le sérum de huit sujets adultes non sélectionnés atteints de leucémie myélogène aiguë, prélevé avant et après la chimiothérapie, a été testé à plusieurs reprises pour évaluer la cytotoxicité spécifique dépendant du complément contre les globules blancs périphériques autochtones obtenus au stade aigu ou lors de la rémission. Au stade aigu, la cytotoxicité a été observée avec les huit sérums, alors qu'au stade de la rémission, aucun des trois sérums testés en parallèle n'a réagi. La cytotoxicité a augmenté après la chimiothérapie, même dans les cas où il n'y a pas eu de rémission.

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DEMONSTRATION OF ANTIBODY-ASSOCIATED CELLULAR CYTOTOXICITY IN PATIENTS WITH ACUTE MYELOGENOUS LEUKEMIA BEFORE AND AFTER CHEMOTHERAPY

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This study demonstrates that a cytotoxic serum reactivity not requiring the presence of complement appears in the sera of patients with acute myelogenous leukemia. The reaction is detected upon short-term incubation of sera *in vitro* with autochthonous mononuclear white blood cells from peripheral venous blood of patients at the acute stage of the disease. This reactivity was demonstrated in 18/18 patients. Generally, the cytotoxicity was low in patients at the acute stage of the disease, but increased after chemotherapy and reached the highest level at the onset of clinical remission or just before. No cytotoxicity could be demonstrated against autochthonous remission white blood cells. The serum activity could be absorbed and eluted from protein A-Sepharose CL-4B and was recovered in the 7S-fraction of the sera after gel filtration on Sephadex G-200 and ion exchange chromatography. This indicates that the demonstrated cytotoxicity is due to immunoglobulins of IgG-class. It is believed that Fc-receptor-bearing cells present in the target cell preparations function as effector cells. The reaction is designated antibody-associated cellular cytotoxicity (AACC).

Cytotoxicity dependent on antibody and mediated by different kinds of leukocytes has been demonstrated *in vitro* against erythroid and non-erythroid, nucleated target cells. In most systems, the antibody is reacted with the target cell and cytotoxicity is mediated via the Fc-receptor on the effector cell (Perlmann and Holm, 1969). It has also been demonstrated that the effector cells can be " armed " with specific antibody (Perlmann and Perlmann, 1970; Parillo and Fauci, 1977). Effector cell activity has been attributed to thymus-independent lymphocytes (MacLennan *et al.*, 1969) adherent cells (Dennert and Lennox, 1973; Jolley *et al.*, 1976), polymorphonuclear leukocytes (Gale and Zighelboim, 1974) and thymus-dependent lymphocytes (Möller, 1971; Fakhri and Hobbs, 1972). More than one type of cell may be active in a single system. Recently, Akira and Takasugi (1977) presented evidence favoring *in vivo* arming of lymphocytes by " natural " antibodies as the mechanism of spontaneous lymphocyte-mediated cytotoxicity.

We have demonstrated specific complement-dependent cytotoxicity against autochthonous leukemia cells in sera from patients with acute myelogenous leukemia (Fäldt and Ankerst, 1977). Under some experimental conditions we also observed a complement-independent cytotoxicity. In the present investigation we demonstrate specific cytotoxicity when heat-inactivated sera from patients are added to cell suspensions consisting of myeloid leukemia cells, lymphoid and adherent cells prepared from whole venous blood by centrifugation on Ficoll-Isopaque solution (spec. grav. 1.077). We present evidence that this form of cytotoxicity is antibody-associated and not complement-dependent, and that apparently the effector cells are autochthonous peripheral white blood cells present in the leukemia target cell preparations. We designate this cytotoxicity antibody-associated cellular cytotoxicity (AACC) since it might not be identical to ADCC (antibody-dependent cellular cytotoxicity).

Cells and sequential sera from 18 patients with acute myelogenous leukemia were tested with regard to this type of specific cytotoxicity.

MATERIAL AND METHODS

Patients

Eighteen patients with acute myelogenous leukemia were studied in the present investigation. Eleven of these were treated at Lund University Hospital and seven at Borås Community Hospital. Some important clinical and laboratory data are summarized in Table I. Four of the patients were given daily treatment with an experimental drug, prednimustine, to which were added two to four injections of vincristine. Fourteen patients were given cyclic chemotherapy with different combinations of cytostatic drugs as specified in Table I.

Peripheral white blood cells from the acute stage of the disease were obtained before therapy was initiated and peripheral white blood cells from the remission stage of the disease were obtained after 3 weeks without chemotherapy. Sera from the patients were obtained before therapy and then sequentially in the course of the disease in periods without drug administration. The time interval between the latest dose of cytostatics and serum collection is indicated in Table I.

Sera

Normal sera from healthy AB blood group donors and sera from patients with acute myelogenous leukemia collected at the acute stage of the disease before therapy and in the course of the disease were stored at −80° C or at −196° C in liquid nitrogen. They were inactivated at 56° C for 30 min before use.

Absorption of sera by protein A

About 1 g of lyophilized preparation of protein A covalently bound to Sepharose CL-4B (Pharmacia,

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TABLE I

CLINICAL AND LABORATORY DATA ON 18 PATIENTS WITH ACUTE MYELOGENOUS LEUKEMIA AT THE TIME OF SERUM COLLECTION

Pat. No., sex and age (years)	Serum [1]	Number of peripheral white blood cells $\times 10^{-3}$ per μl and % of blast cells		% of blast cells in the bone marrow	Clinical stage of the disease	Chemotherapy	
						Schedule [4]	Days after latest dose
3	PA-3	7.0	70%	60%	Before treatment	—	—
Male, 49	PD-3	5.6	0%	14%	After 10 weeks of treatment without remission	TA×3	8
16	PA-16	13.2	43%	98%	Before treatment	—	—
Male, 39	PC-16	2.0	0%	<5%	In complete remission after 6 weeks of treatment	0×2+prednimustine daily	1
19	PA-19	87.0	75%	90%	Before treatment	—	—
Male, 25	PI-19	7.2	3%	44%	In relapse after 32 weeks of treatment	POMP×3+TA×1+TRA×2+POMP×2	11
	PM-19	11.4	48%	78%	In relapse after 41 weeks of treatment	+POMP×1	14
	PN-19	11.0	38%	83%	In relapse after 43 weeks of treatment	+TA×1+POMP×2	10
22	PA-22	20.0	15%	56%	Before treatment	—	—
Female, 24	PG-22	12.0	ND [2]	ND	After 19 weeks of treatment without remission	0×4+prednimustine daily	1
26	PA-26	2.9	14%	76%	Before treatment	—	—
Male, 24	PD-26	3.5	3%	30%	After 19 weeks of treatment without remission	TRAP×7+COAP×1	14
28	PA-28	20.0	35%	85%	Before treatment	—	—
Male, 37	PB-28	2.8	0%	ND	After 2 weeks of treatment	TOMP×1	10
	PC-28	5.1	0%	<5%	In complete remission after 34 weeks of treatment	+TOMP×1+POMP×1+COA×5	27
	PD-28	6.2	0%	<5%	In complete remission after 62 weeks of treatment	+RA×1+COA×1	25
	PE-28	22.7	11%	12%	In relapse after 72 weeks of treatment	+COA×1	34
	PF-28	6.7	0%	ND	In relapse after 95 weeks of treatment	+RA×2+COA×5	10
29	PA-29	88.0	47%	83%	Before treatment	—	—
Male, 50	PC-29	10.1	5%	ND	After 2 weeks of treatment	TRAP×1	9
	PG-29	4.0	0%	<5%	In complete remission after 18 weeks of treatment	+TRAP×3+COAP×4	10
	PH-29	2.2	0%	20%	In relapse after 26 weeks of treatment	+TRAP×2	7
30	PA-30	30.0	80%	57%	Before treatment	—	—
Female, 63	PB-30	5.3	9%	51%	After 3 weeks of treatment	TA×1	18
	PC-30	4.1	ND	6%	After 10 weeks of treatment without remission	+TA×2	7
31	PA-31	19.0	34%	77%	Before treatment	—	—
Male, 68	PC-31	10.2	0%	<5%	In complete remission after 10 weeks of treatment	0+2+VAMP×2	9
	PD-31	2.5	5%	89%	In relapse after 32 weeks	+POMP×2+TA×4+POMP×1	10

(Cont'd opposite)

Pat. No., Sex and age (years)	Serum [1]	Number of peripheral white blood cells $\times 10^{-3}$ per μl and % of blast cells		% of blast cells in the bone marrow	Clinical stage of the disease	Chemotherapy	
						Schedule [4]	Days after latest dose
32	PA-32	149.0	95%	97%	Before treatment	—	—
Male, 44	PB-32	3.8	6%	89%	After 6 weeks of treatment	TA×2	18
	PE-32	4.4	0%	<5%	In complete remission after 19 weeks of treatment	+TRA×1+POMP×1+COA×1+TOMP×2	1
35	PA-35	49.0	15%	68%	Before treatment	—	—
Female, 36	PB-35	3.3	0%	5%	After 4 weeks of treatment	TRAP×2	7
	PC-35	2.1	0%	<5%	In complete remission after 7 weeks of treatment	+TRAP×1	10
	PF-35	2.6	0%	<5%	In complete remission after 32 weeks of treatment	+TRAP×4+COAP×2	14
	PI-35	1.2	20%	ND	In relapse after 63 weeks of treatment	+TRAP×2+COAP×2+TRAP×4	2
36	PA-36	15.4	24%	Dry tap [3]	Before treatment	—	—
Female, 31	PC-36	2.0	0%	<5%	In complete remission after 12 weeks of treatment	TA×4	13
	PD-36	9.6	0%	<5%	In complete remission after 24 weeks of treatment	+TRA×1+POMP×1	28
39	PA-39	3.4	6%	46%	Before treatment	—	—
Female, 50	PB-39	4.9	1%	26%	After 3 weeks of treatment	0×2+prednimustine daily	1
	PC-39	1.9	0%	30%	After 6 weeks of treatment without remission	0×3+prednimustine daily	1
41	PA-41	47.7	89%	90%	Before treatment	—	—
Male, 30	PE-41	8.1	1%	8%	After 11 weeks of treatment without remission	TRAP×3+COAP×1	3
42	PA-42	15.5	ND	92%	Before treatment	—	—
Female, 22	PB-42	0.9	ND	ND	After 10 weeks of treatment	TRAP×5	5
	PD-42	3.7	ND	ND	In complete remission after 19 weeks of treatment	+TRAP×2+COAP×1	18
	PE-42	2.1	ND	72%	In relapse after 30 weeks of treatment	+COAP×1+TRAP×2	16
	PF-42	29.9	81%	ND	In relapse after 72 weeks of treatment	+COAP×1+VAMP×9+prednimustine daily	5
49	PA-49	85.0	85%	93%	Before treatment	—	—
Female, 23	PE-49	4.2	0%	<5%	In complete remission after 23 weeks of treatment	RA×1+TRA×2+TA×1+TRA×1+TA×1	6
50	PA-50	75.0	ND	70%	Before treatment	—	—
Female, 30	PB-50	0.6	4%	ND	After 5 weeks of treatment	RA×2	6
	PC-50	1.1	0%	<5%	In complete remission after 7 weeks of treatment	+RA×1	2
	PD-50	6.0	ND	<5%	In complete remission after 9 weeks of treatment	RA×3	16
	PE-50	9.9	ND	<5%	In complete remission after 14 weeks of treatment	+RA×1	21
51	PA-51	23.5	60%	35%	Before treatment	—	—
Female, 54	PB-51	8.6	ND	22%	After 3 weeks of treatment without remission	0×2+prednimustine daily	1

[1] Sera were designated as follows: PA, patients' sera taken before any treatment. PB-PN, patients' sera taken at various stages of the disease during chemotherapy. The letters are followed by the patients' numbers. — [2] Not done. — [3] Unsuccessful bone marrow aspiration as a consequence of packed cells in bone marrow. — [4] Abbreviations: TA, thioguanine-cytarabine; O, vincristine; Prednimustine, chlorambucil-rednisolone; TRAP, thioguanine-daunorubicin-cytarabine-prednisolone; COAP, cyclophosphamide-vincristine-cytarabine-prednisolone; TRA, thioguanine-daunorubicin-cytarabine; POMP, mercaptopurine vincristine-methothrexate-prednisolone; COA, cyclophosphamide-vincristine-cytarabine; TOMP, thioguanine-vincristine-methothrexate-prednisolone; RA, daunorubicin-cytarabine.

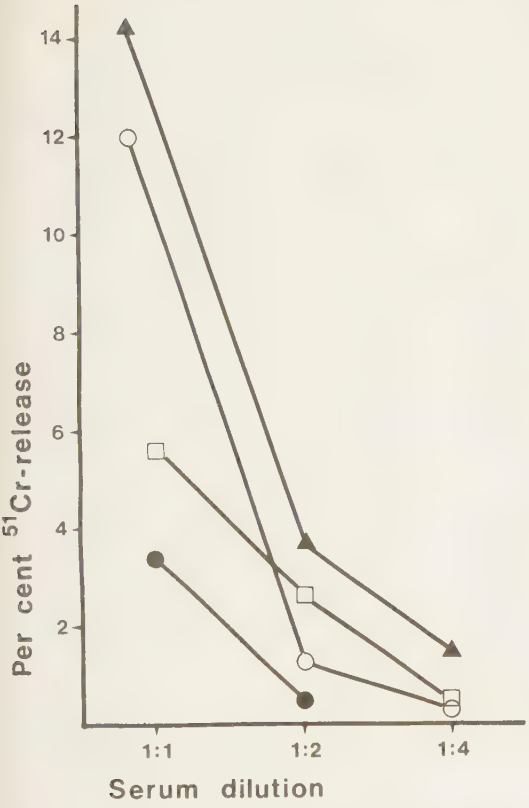

FIGURE 1 — Titration of sera from four patients with acute myelogenous leukemia expressing antibody-associated cellular cytotoxicity as demonstrated by the ^{51}Cr-release technique. The difference in percentage of ^{51}Cr-release between the patients' sera and normal sera of the corresponding dilution is indicated on the ordinate. ▲——▲, Serum No. PI-19. ○——○, Serum No. PC-16. □——□, Serum No. PG-29. ●——●, Serum No. PF-35.

Uppsala, Sweden) was swollen in PBS (1.5 mM KH_2PO_4, 6 mM $Na_2HPO_4 \cdot 2H_2O$, 137 mM NaCl, 3 mM KCl, 1 mM $CaCl_2 \cdot 2H_2O$, 0.5 mM $MgCl_2 \cdot 6H_2O$) and packed in a 1.25 × 4 cm chromatographic column producing about 5.0 ml gel bed with a human IgG binding capacity of approximately 25 mg IgG/ml. The columns were kept at 4° C and before each chromatographic separation they were washed with PBS. One ml of each serum sample was applied to the column and washed through with PBS. The absorbed material was eluted with 0.1 M glycine·HCl, pH 3.1. The effluent and the eluate were dialyzed against PBS and concentrated to the original serum volume on XM-100 membrane (Amicon, Lexington, USA) by ultrafiltration.

Chromatographic separation of sera

Sera from two patients with acute myelogenous leukemia and sera from two healthy AB blood group donors were separated chromatographically. The combination of Sephadex G-200 gel filtration and DEAE ion-exchange cellulose chromatography was used for the preparation of immunoglobulins corresponding to 7S and 19S fractions, respectively. Two ml of each serum were applied to a column (2.5 × 90 cm) of Sephadex G-200 (Pharmacia, Uppsala, Sweden). Filtration with phosphate-buffered saline (30 mM phosphate, pH 7.2, 0.12 M NaCl) was performed at 4° C. The fractions of the first peak were pooled and concentrated by lyophilizing. Before use they were dissolved in 1 ml PBS and dialyzed against PBS. The pooled fractions of the second peak were concentrated to the original serum volume, dialyzed against 20 mM phosphate buffer, pH 8.0 and applied to a column (2.2 × 15 cm) of Whatman DE 32 cellulose (W&R Balston Ltd., England). Elution was performed with 20 mM phosphate buffer, pH 8.0 and concentrated by lyophilization. Before use they were dissolved in PBS of the half of the original volume and dialyzed against PBS.

The content of immunoglobulins in the original sera and their fractions corresponding to 7S and 19S immunoglobulins were determined in double immunodiffusion tests with specific rabbit anti-human IgA, IgG and IgM sera (AS Dakopatts, Copenhagen, Denmark) and by the technique of Laurell (1972). The amount of immunoglobulins after chromatographic separation was not significantly decreased as compared to the amounts of unfractionated sera. Neither contamination of the fractions corresponding to 19S immunoglobulins with IgA or IgG nor contamination of fractions corresponding to 7S immunoglobulins with IgM could be demonstrated.

Cell suspensions

Peripheral white blood cells from patients with acute myelogenous leukemia in the acute stage of the disease before therapy and remission white blood cells from 5 of the patients harvested within 4 months of onset of remission were used in the experiments. The cells were separated from heparinized whole venous blood by centrifugation on Ficoll-Isopaque (density 1.077 g/cm^3) as previously described (Fäldt and Ankerst, 1977). The cells in the interphase, consisting of myeloid leukemia cells, lymphoid and adherent cells were frozen and stored in liquid nitrogen and after thawing they were used in the experiments.

In vitro *assay of antibody-associated cellular cytotoxicity*

After thawing in a 40° C water bath the cell suspensions were washed twice in PBS with 10% fetal calf serum and 2% Hepes at 37° C and room temperature (20-24° C), respectively. Appropriate labelling procedure with ^{51}Cr in the form of sodium chromate (New England Nuclear, Boston, Mass., USA, spec. act. 200-500 Ci/g) varied from patient to patient. The incubation time with ^{51}Cr was either 3 h or 15-16 h at 37° C. Cell suspensions from each patient were incubated and the alternative giving optimal sensitivity in testing was chosen for each patient.

The 3-h incubation was performed at 37° C with 1.5×10^6 viable cells suspended in 2 ml PBS with 5%

TABLE II

ANTIBODY-ASSOCIATED CELLULAR CYTOTOXICITY OF SEQUENTIAL AUTOCHTHONOUS SERA AGAINST WHITE BLOOD CELLS OF PATIENTS WITH ACUTE MYELOGENOUS LEUKEMIA

Experiment	Serum [1]	% ^{51}Cr-release ± SE	Difference compared to [2]		Weeks after onset of treatment	Clinical stage of the disease
			Control serum	Serum from acute stage		
1. Pat. No. 3	AB-3	12.1±0.5	—	—	—	—
	PA-3	13.7±0.8	1.6 $p>0.5$	—	0	Acute stage
	PD-3	16.6±0.3	4.5 $p<0.0005$	2.9 $p<0.01$	10	No remission
2. Pat. No. 16	AB-3	15.0±0.2	—	—	—	—
	PA-16	12.9±0.1	−2.1 $p<0.0005$	—	0	Acute stage
	PC-16	27.1±0.6	12.1 $p<0.0005$	14.2 $p<0.0005$	6	Remission
3. Pat. No. 19	AB-13	20.1±1.6	—	—	—	—
	PA-19	25.4±1.4	5.3 $p<0.05$	—	0	Acute stage
	PM-19	30.0±1.6	9.9 $p<0.01$	4.6 $p>0.05$	41	Relapse
	PN-19	28.3±0.8	8.2 $p<0.01$	2.9 $p>0.05$	43	Relapse
4. Pat. No. 22	AB-10	19.6±0.4	—	—	—	—
	PA-22	21.8±1.9	2.2 $p>0.05$	—	0	Acute stage
	PG-22	27.9±0.9	8.3 $p<0.01$	6.1 $p<0.05$	19	No remission
5. Pat. No. 26	AB-11	24.5±0.6	—	—	—	—
	PA-26	26.1±0.9	1.6 $p>0.05$	—	0	Acute stage
	PD-26	31.1±0.5	6.6 $p<0.0005$	5.0 $p<0.01$	19	No remission
6. Pat. No. 28	AB-20	6.1±0.2	—	—	—	—
	PB-28	10.1±0.5	4.0 $p<0.01$	—	2	No remission
	PC-28	9.0±0.1	2.9 $p<0.0005$	—	34	Remission
	PD-28	8.9±0.7	2.8 $p<0.01$	—	62	Remission
	PE-28	6.4±0.8	0.3 $p>0.05$	—	72	Relapse
	PF-28	10.0±1.0	3.9 $p<0.01$	—	95	Relapse
7. Pat. No. 29	AB-10	17.8	—	—	—	—
	AB-12	15.2 ⟩ 16.5±0.9				
	PA-29	16.2±1.4	−0.3 $p>0.05$	—	0	Acute stage
	PC-29	19.1±0.3	2.6 $p<0.05$	2.9 $p>0.05$	2	No remission
	PG-29	24.6±0.6	8.1 $p<0.0005$	8.4 $p<0.01$	18	Remission
	PH-29	19.7±0.8	3.2 $p<0.05$	3.5 $p<0.05$	26	Relapse
8. Pat. No. 30	AB-22	16.8±0.6	—	—	—	—
	PA-30	17.9±0.3	1.1 $p>0.05$	—	0	Acute stage
	PB-30	19.7±0.5	2.9 $p<0.05$	1.8 $p<0.05$	3	No remission
	PC-30	20.8±0.9	4.0 $p<0.05$	2.9 $p<0.05$	10	No remission
9. Pat. No. 31	AB-15	6.7±0.3	—	—	—	—
	PA-31	6.3±0.2	−0.4 $p>0.05$	—	0	Acute stage
	PC-31	10.8±0.3	4.1 $p<0.0005$	4.5 $p<0.0005$	10	Remission
	PD-31	8.3±0.2	1.6 $p<0.01$	2.0 $p<0.01$	32	Relapse
10. Pat. No. 32	AB-10	12.7	—	—	—	—
	AB-12	14.5 ⟩ 13.6±1.2				
	PA-32	21.2±1.4	7.6 $p<0.01$	—	0	Acute stage
	PB-32	22.0±3.2	8.4 $p<0.01$	0.8 $p>0.05$	6	No remission
	PE-32	28.3±1.1	14.7 $p<0.0005$	7.1 $p<0.01$	19	Remission
11. Pat. No. 35	AB-10	3.3	—	—	—	—
	AB-12	3.2 ⟩ 3.2±0.2				
	PA-35	3.9±0.1	0.7 $p<0.05$	—	0	Acute stage
	PB-35	4.8±0.3	1.6 $p<0.01$	0.9 $p<0.05$	4	No remission
	PC-35	4.3±0.3	1.1 $p<0.01$	0.4 $p>0.05$	7	Remission
	PF-35	6.8±0.6	3.6 $p<0.0005$	2.9 $p<0.01$	32	Remission
12. Pat. No. 36	AB-17	5.9±0.5	—	—	—	—
	PA-36	6.8±0.8	0.9 $p>0.05$	—	0	Acute stage
	PC-36	8.2±0.2	2.3 $p<0.01$	1.4 $p>0.05$	12	Remission
	PD-36	10.3±0.6	4.4 $p<0.01$	3.5 $p<0.05$	24	Remission
13. Pat. No. 39	AB-13	14.3±0.3	—	—	—	—
	PA-39	14.8±0.3	0.5 $p>0.05$	—	0	Acute stage
	PB-39	15.6±0.4	1.3 $p<0.05$	0.8 $p>0.05$	3	No remission
	PC-39	16.3±0.3	2.0 $p<0.01$	1.5 $p<0.01$	6	No remission

(Cont'd overleaf)

Experiment	Serum [1]	% ^{51}Cr-release±SE	Difference compared to [2]		Weeks after onset of treatment	Clinical stage of the disease
			Control serum	Serum from acute stage		
14. Pat. No. 41	AB-17	7.9±0.5	—	—	—	—
	PA-41	8.0±0.4	0.1 $p>0.05$	—	0	Acute stage
	PE-41	10.1±0.4	2.2 $p<0.05$	2.1 $p<0.05$	11	No remission
15. Pat. No. 42	AB-22	4.7±0.1	—	—	—	—
	PA-42	6.6±0.3	1.9 $p<0.01$	—	0	Acute stage
	PB-42	7.2±0.2	2.5 $p<0.0005$	0.6 $p>0.05$	10	No remission
	PD-42	5.6±0.2	0.9 $p<0.05$	−1.0 $p<0.05$	19	Remission
	PE-42	5.8±0.3	1.1 $p<0.01$	−0.8 $p<0.05$	30	Relapse
	PF-42	9.9±0.4	5.2 $p<0.0005$	3.3 $p<0.01$	72	Relapse
16. Pat. No. 49	AB-20	10.8±0.6	—	—	—	—
	PA-49	20.4±0.8	9.6 $p<0.0005$	—	0	Acute stage
	PE-49	20.4±0.4	9.6 $p<0.0005$	0.0 $p>0.05$	23	Remission
17. Pat. No. 50	AB-20	6.3 > 6.4±0.2				
	AB-22	6.5	—	—	—	—
	PA-50	8.6±0.4	2.2 $p<0.01$	—	0	Acute stage
	PB-50	12.0±0.3	5.6 $p<0.0005$	3.4 $p<0.01$	5	No remission
	PC-50	9.1±0.3	2.7 $p<0.0005$	0.5 $p>0.05$	7	Remission
	PD-50	7.5±0.3	1.1 $p<0.05$	−1.1 $p<0.05$	9	Remission
	PE-50	7.0±0.4	0.6 $p>0.05$	−1.6 $p<0.05$	14	Remission
18. Pat. No. 51	AB-20	5.4±0.1	—	—	—	—
	PA-51	8.8±0.4	3.4 $p<0.01$	—	0	Acute stage
	PB-51	7.6±0.2	2.2 $p<0.0005$	−1.2 $p<0.05$	3	No remission

[1] Sera designated as follows: AB, sera of healthy donors of blood group AB followed by the donor's number; PA, patients' sera taken before treatment with cytostatics; PB-PN, patients' sera taken at various stages of the disease. The letters are followed by the patients' numbers. — [2] The probability that the recorded difference is due to chance was calculated by Student's t-test.

fetal calf serum, 1% Hepes and 1/2 mCi/ml ^{51}Cr. After the incubation the fluid was removed and 1 ml of fetal calf serum containing 2 drops (50 μl) Varidase (Lederle Laboratories, Wayne, N.J., USA) (10,000 IU/ml) was added to the cells. After about 30 sec, 10 ml PBS with 10% fetal calf serum and 2% Hepes were added and the cell suspensions left at 4° C for 19-22 h. Before testing the supernatant was replaced with 5 ml PBS with 10% heat-inactivated fetal calf serum and 2% Hepes. After addition of 0.5 ml Varidase the cell suspensions were left at 37° C in a water bath for 15-30 min. Five ml of cold (4° C) PBS with 10% heat-inactivated fetal calf serum and 2% Hepes were added and the cell suspensions spun at 150 *g* for 5 min. The cells were suspended in PBS with 10% heat-inactivated AB serum and 1 mM $MgCl_2$, not containing $CaCl_2$. Aliquots of 1.0 ml cell suspension containing 7.5×10^4 viable cells were spun at 400 *g* for 1 min, and sedimented cells resuspended in test serum of the desired dilution. All experiments were performed in triplicate sets. A final concentration of 2.5×10^4 cells/0.1 ml of test serum was incubated for 15 min at room temperature (20-24° C). After addition of 4 ml PBS with 1 mM $MgCl_2$, not containing $CaCl_2$, and 10% heat-inactivated fetal calf serum cooled to 4° C, the cells were spun down at 700 *g* for 5 min. After 45 min at 4° C, 1 ml of the supernatant and the original tubes were counted in a gamma counter.

Alternatively the incubation was prolonged for 15-16 h at 37° C. After the incubation two drops (50 μl) Varidase were added to the cell suspensions, which were gently shaken for approximately 30 sec. Five ml of PBS with 10% heat-inactivated fetal calf serum and 2% Hepes and 0.5 ml Varidase were added and the cell suspensions centrifugated at 150 *g* for 5 min. The cell suspensions were washed once with PBS containing 10% heat-inactivated fetal calf serum with 2% Hepes and resuspended in PBS with 1mM $MgCl_2$, not containing $CaCl_2$, and 10% heat-inactivated AB serum. The cell suspensions were then handled in the same way as in the tests described above.

The percentage of isotope release was calculated as follows:

$$\frac{\text{Radioactivity in 1.0 ml of supernatant} \times 4.1 \times 100}{\text{Total radioactivity/tube}}$$

The statistical significance of the recorded differences between the AACC activity of patients' sera and reference ^{51}Cr-release caused by control AB-sera was calculated by Student's t-test. Significance of differences between sequential sera from a given patient and the serum obtained from the same patient in the acute stage was similarly calculated and presented in Table II.

In some cases the relative rise in specific cytotoxicity is discussed in the text. Relative rise in specific cytotoxicity means the ratio between the cytotoxicity of the patient's serum showing the highest cytotoxicity and the cytotoxicity of the serum obtained before initiation of therapy.

RESULTS

Eighteen patients with acute myelogenous leukemia were studied and the results are summarized in Tables I-V and Figure 1. Data reflecting some important laboratory and clinical parameters of the disease at the time of serum collection are presented in Table I.

Sera obtained at the acute stage of the disease before initiation of therapy and sera collected after chemotherapy were tested for AACC activity against autochthonous peripheral white blood cells. Their cytotoxic activities were compared to the ^{51}Cr-release level in the presence of reference control AB-sera. The cells harvested in the acute stage of the disease were stored in liquid nitrogen until use.

Specific antibody-associated cellular cytotoxicity was demonstrated in 18/18 patients with acute myelogeneous leukemia (Table II). The cytotoxic activity of patients' sera taken before chemotherapy was usually low or statistically not significant as compared to control AB-sera. After treatment of the patients with cytostatic drugs the cytotoxic activity increased in sequential sera to statistically highly significant levels independent of the type of chemotherapy used. In 11 remission patients the cytotoxic activity was highest just before remission or in early remission. Later on in the remission stage, the cytotoxic activities fell and became lowest at the time of early relapse. In the relapse stage significant cytotoxic activities above control level could again be demonstrated after intensive chemotherapy in the sera of patients 19, 28 and 42. Even in seven patients who did not enter remission, significant AACC-activity could be detected after chemotherapy. The relative rise in AACC-activities of sera from these patients was, however, lower than the relative rise in AACC activities of sera from patients who went into remission. The cytotoxic activity was generally highest in undiluted sera, still demonstrable at dilution 1/2, and, with few exceptions, decreased to insignificant levels at dilution 1/4 (Fig. 1). Patients 49 and 51 showed no increase in AACC activity after chemotherapy; however, serum from patient No. 49 demonstrated highly significant AACC activity even before initiation of therapy.

The specificity of the cytotoxicity was checked by parallel testing of five patients' sera against autochthonous peripheral white blood cells obtained at both the acute and remission stages of the disease (Table III). The experimental conditions were identical when white blood cells from the acute and remission stages were tested and the cell suspensions were tested simultaneously. Of five sera showing highly significant AACC activities against autochthonous cells harvested during the acute stage of the disease, four expressed no AACC activity against white blood cells harvested from the patients early in the remission stage of the disease. One of five patients' sera showed significant AACC activity against autochthonous white blood cells early in the remission stage, but a few months later the same serum showed no AACC activity against remission cells harvested from the patient later in the remission stage.

In order to demonstrate that the AACC activity is immunoglobulin-dependent, eight patients' sera were absorbed with protein A by passage through a column containing protein A coupled to Sepharose CL-4B (Table IV). After absorption to protein A, the AACC activity was lost in each of eight patients' sera which had significant AACC activity against autochthonous leukemia cell suspensions before absorption. In six cases the absorbed activity was eluted from the column by glycine·HCl, pH 3.1. After concentration of the eluates to the original

TABLE III

STUDIES OF ANTIBODY-ASSOCIATED CELLULAR CYTOTOXICITY OF AUTOCHTHONOUS SERA AGAINST WHITE BLOOD CELLS OBTAINED AT ACUTE AND REMISSION STAGES OF ACUTE MYELOGENOUS LEUKEMIA

Experiment	Serum [1]	Target cells: White blood cells from the acute stage of the disease		White blood cells from the same patient at the beginning of remission		White blood cells from the same patient some months later in remission	
		% ^{51}Cr-release ±SE	Difference compared to [3] control serum	% ^{51}Cr-release ±SE	Difference compared to control serum	% ^{51}Cr-release ±SE	Difference compared to control serum
1. Pat. No. 28	AB-20	5.7±0.2	—	4.1±0.3	—	ND [3]	
	PD-28	10.6±0.1	4.9 $p<0.0005$	4.1±0.0	0 $p>0.05$		
2. Pat. No. 35	AB-15	4.9±0.4	—	7.0±0.3	—	8.6±0.2	—
	PI-35	9.9±0.2	5.0 $p<0.0005$	8.7±0.5	1.7 $p<0.05$	9.3±0.5	0.7 $p>0.05$
3. Pat. No. 36	AB-16	7.9±0.1	—	7.3±0.5	—	5.1±0.5	—
	PC-36	9.9±0.3	2.0 $p<0.01$	7.9±0.9	0.6 $p>0.05$	4.8±0.5	−0.3 $p>0.05$
4. Pat. No. 49	AB-20	13.2±0.3	—	6.0±0.3	—	ND	
	PE-49	17.5±0.3	4.3 $p<0.0005$	5.6±0.1	−0.4 $p>0.05$		
5. Pat. No. 50	AB-20	5.8±0.2	—	5.6±0.1	—	ND	
	PB-50	9.3±0.1	3.5 $p<0.0005$	5.3±0.2	−0.3 $p>0.05$		

[1] See footnote [1], Table I. — [2] see footnote [2], Table II. — [3] See footnote [2], Table I.

TABLE IV

ANTIBODY-ASSOCIATED CELLULAR CYTOTOXICITY OF AUTOCHTHONOUS SERA FROM EIGHT PATIENTS WITH ACUTE MYELOGENOUS LEUKEMIA AS COMPARED TO THEIR EFFLUENTS AND ELUATES AFTER ABSORPTION ON A PROTEIN A COLUMN

Experiment	Serum [1]	Untreated serum		Absorbed serum		Eluate	
		% ^{51}Cr-release ±SE	Difference compared to [2] control serum	% ^{51}Cr-release ±SE	Difference compared to control serum	% ^{51}Cr-release ±SE	Difference compared to control serum
1. Pat. No. 28	AB-20	9.0±0.4	—	8.5±0.7	—	9.2±0.4	—
	PF-28	10.7±0.1	1.7 $p<0.01$	6.8±0.7	−1.7 $p>0.05$	10.3±0.3	1.1 $p<0.05$
2. Pat. No. 29	AB-12	6.3±0.2	—	7.2±0.2	—	6.3±0.5	—
	PG-29	10.5±0.3	4.2 $p<0.0005$	5.4±0.3	−1.8 $p<0.01$	10.4±0.3	4.1 $p<0.01$
3. Pat. No. 31	AB-15	20.6±1.0	—	14.5±0.4	—	16.3±0.5	—
	PC-31	28.4±1.2	7.8 $p<0.01$	15.6±2.2	1.1 $p>0.05$	22.5±2.2	6.2 $p<0.05$
4. Pat. No. 32	AB-12	18.7±0.7	—	19.7±0.5	—	ND [3]	
	PB-32	28.5±0.8	9.8 $p<0.0005$	14.3±0.4	−5.4 $p<0.01$		
5. Pat. No. 35	AB-15	6.9±0.4	—	4.8±0.6	—	5.1±0.1	—
	PF-35	13.4±0.8	6.5 $p<0.01$	4.9±0.1	0.1 $p>0.05$	6.4±0.5	1.3 $p<0.05$
6. Pat. No. 36	AB-17	2.9±0.1	—	3.2±0.1	—	ND	
	PB-36	6.0±0.2	3.1 $p<0.0005$	2.1±0.1	−1.1 $p<0.01$		
7. Pat. No. 49	AB-20	17.0±0.3	—	16.0±0.3	—	17.2±0.1	—
	PE-49	24.4±0.5	7.4 $p<0.0005$	16.4±0.6	0.4 $p>0.05$	24.3±1.2	7.1 $p<0.01$
8. Pat. No. 50	AB-20	9.0±0.3	—	8.4±0.1	—	7.8±0.1	—
	PB-50	14.5±0.4	5.5 $p<0.0005$	8.7±0.4	0.3 $p>0.05$	9.4±0.5	1.6 $p<0.05$

[1] See footnote [1], Table I. — [2] See footnote [2], Table II. — [3] See footnote [2], Table I.

sample volume, significant cytotoxicity could be detected in all six cases, indicating that the AACC activity in the sera is antibody-associated.

To check further that the AACC activity in the patients' sera is antibody-associated and to study its association to immunoglobulin classes, two patients' sera and two reference control AB-sera were fractionated chromatographically on Sephadex G-200 and DEAE-cellulose. In both fractionated sera of patients with acute myelogenous leukemia the cytotoxicity could be detected in the fractions containing IgG, while no specific cytotoxicity could be detected in the IgM-containing fractions (Table V).

DISCUSSION

Cytotoxic activities requiring immunoglobulins and Fc-receptor-bearing white blood cells have been studied in several systems and antibody-dependent lymphocyte killing of acute myelogenous leukemia cells has been demonstrated (Hersey *et al.*, 1973). In the latter study, however, allogeneic material was used and the specificity of the findings may therefore be questioned.

We have previously demonstrated specific antibodies against autochthonous leukemia cells in patients with acute myelogenous leukemia (Fäldt and Ankerst, 1977). Whereas the humoral comp-

TABLE V

ANTIBODY-ASSOCIATED CELLULAR CYTOTOXICITY OF AUTOCHTHONOUS SERA FROM TWO PATIENTS WITH ACUTE MYELOGENOUS LEUKEMIA AS COMPARED TO THEIR 7S- AND 19S-FRACTIONS OBTAINED AFTER CHROMATOGRAPHIC SEPARATION ON SEPHADEX G-200 AND DE 32 CELLULOSE

Experiment	Serum [1]	Untreated serum		7S-fraction		19S-fraction	
		% ^{51}Cr-release ±SE	Difference compared to [2] control serum	% ^{51}Cr-release ±SE	Difference compared to control serum	% ^{51}Cr-release ±SE	Difference compared to control serum
1. Pat. No. 19	AB-22	13.6±0.1	—	11.3±0.1	—	11.1±0.5	—
	PI-19	21.6±0.8	8.0 $p<0.0005$	16.2±0.2	4.9 $p<0.0005$	10.7±0.7	−0.4 $p>0.05$
2. Pat. No. 35	AB-20	4.2±0.3	—	4.7±0.3	—	3.5±0.6	
	PF-35	8.1±0.3	3.9 $p<0.01$	7.8±0.2	3.1 $p<0.0005$	3.2±0.1	−0.3 $p>0.05$

[1] See footnote [1], Table I. — [2] See footnote [2], Table II.

lement-dependent cytotoxicity could be detected only under particular experimental conditions, it was noticed that some cytotoxic activity could be detected in some patients' sera also in the absence of heterologous complement when sera were added to mononuclear white blood cells obtained after Ficoll-Isopaque separation. The present investigation indicates that this cytotoxicity is mediated by non-adherent cells present in the leukemia cell preparations. The effector cells have not yet been identified or characterized but it has been shown that removal of adherent cells by passage through a nylon wool column or by incubation in plastic Petri dishes did not reduce the cytotoxicity. In the present investigation we describe this form of cytotoxicity as depending on specific serum antibody directed against autochthonous leukemia cells and the presence of autochthonous target and effector cells represented in the band of mononuclear peripheral white blood cells collected in the interphase after Ficoll-Isopaque separation. We believe that this reaction is mediated via Fc-receptor-bearing cells in the same way as the ADCC reaction. However, the presently described reaction differs from ADCC in two major ways: (1) it occurs only with undiluted sera or at low serum dilutions (2) it is very rapid, being detectable after incubation for 15 min at room temperature (20-24° C). Therefore, we designate this cytotoxicity AACC = antibody-associated cellular cytotoxicity, not to be confused with ADCC = antibody-dependent cellular cytotoxicity at the present time.

Specific cytotoxicity using the AACC assay was demonstrated in 18/18 patients with acute myelogenous leukemia. The cytotoxicity was generally low or statistically not significant before initiation of therapy, but it appeared even after a very short period of chemotherapy and was found to be highest just before or during early clinical remission. During continued chemotherapy the AACC activity in sequential patient sera fell to lower levels and was lowest or not significant in the early relapse stage. Later in the relapse stage of the disease, AACC activity could be detected again in the sera of patients 19, 28 and 42 after intensive chemotherapy. AACC activity could also be detected in the sera obtained after chemotherapy of those 7 patients who did not enter the remission stage. However, the relative rise in cytotoxicity of their sequential sera during chemotherapy was lower compared to that of the patients who entered the remission stage. The demonstration that sera tested simultaneously on autochthonous remission white blood cells and on cells obtained at the acute stage of the disease had an AACC effect only on the latter cells in 4/5 cases indicates that the cytotoxic activity of the sera is leukemia-specific and that the leukemia-antigen-bearing cells are not present in the peripheral blood during remission or are greatly reduced in number. In patient No. 35, AACC activity could be demonstrated in the serum PI-35 when tested with white blood cells obtained early in clinical remission, but no activity was detectable when tested with remission cells obtained some months later in the remission stage. Analogous results were previously obtained in studies of complement-dependent cytotoxicity in patients with acute myelogenous leukemia (Fäldt and Ankerst, 1977). Several patients' sera have been tested against autochthonous leukemia cells with regard to both complement-dependent cytotoxicity and AACC activity, with concordant results.

Protein A is known to react and bind mainly to IgG-immunoglobulin, but minor subclasses of IgA and IgM are also bound. In order to show that the AACC activity in the sera is immunoglobulin-associated, 8 patients' sera were passed through a column containing protein A coupled to Sepharose CL-4B. The AACC activity was removed from all of 8 sera absorbed and was recovered in the eluates of 6/6 sera. This indicates that the AACC activity in the patients' sera is associated with immunoglobulin. In an attempt to further characterize these immunoglobulins and to exclude non-immunoglobulin-associated cytotoxicity, 2 patients' sera and 2 control sera were fractionated using filtration on Sephadex G 200 and DEAE-cellulose. The AACC activity was detected in the IgG-containing fractions, while no specific activity was found in the IgM-containing fractions. Although these findings indicate that IgG probably is the main immunoglobulin involved, it cannot be excluded that specific IgM-antibodies may be involved in this type of reaction. The fact that the separated sera were taken some months after onset of the disease might explain the association of the cytotoxic activity with IgG-antibodies. With more extensive studies it may be shown that IgM can posses AACC activity, since it has been demonstrated that some T lymphocytes have receptors for IgM and that they can function as effector cells through their Fc-receptors in some systems (Möller, 1971; Fakhri and Hobbs, 1972).

As previously discussed in relation to the complement-dependent cytotoxicity, the appearance of specific cytotoxic antibodies in patients with acute myelogenous leukemia subjected to chemotherapy may be due to reduced tumor burden. This would diminish the absorbing capacity of the leukemia-antigen-bearing cells and leave free antibody in excess. Diminished production of soluble leukemia antigen would lead to a reduction in the formation of antigen-antibody complexes and consequently excess of free antibody. These explanations anticipate that the antibody-forming cells are not seriously affected by the therapy.

Introduction of the AACC assay in the study of specific antibodies in patients with acute myelogenous leukemia has many advantages. Firstly, the test system is completely autochthonous, thereby eliminating interpretation difficulties such as the participation of anti-HLA-antibodies and other antibodies that must be excluded in the allogeneic situation. Secondly, there is no need for heterologous complement in order to study cytotoxic antibodies in these patients. The finding of a suitable complement source is a problem in many situations (Fäldt and Ankerst, 1977). Thirdly, there is no need for exogenous effector cells as in the many variants of the ADCC assay. A possible difficulty in comparing different patients is that the proportions of effector cells are likely to vary between patients.

The biological significance of the AACC activity remains to be clarified. One may speculate that the AACC activity may be an important *in vivo* cytotoxic mechanism, functioning for instance in immunological defense against leukemia. In contrast to many other assays, the present experimental conditions are similar to those *in vivo:* only autochthonous target cells, effector cells and antibodies are involved.

In the study of chemotherapy of acute myelogenous leukemia with different combinations of cytostatic drugs, varying degrees of immunosuppressive effects and various immunotherapeutic procedures, it would be extremely valuable to have available tests measuring specific leukemia antibodies. The present investigation shows that the AACC assay can be used for such purposes in the study of patients with acute myelogenous leukemia.

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MISE EN ÉVIDENCE D'UNE CYTOTOXICITÉ CELLULAIRE ASSOCIÉE A L'ANTICORPS DANS DES CAS DE LEUCÉMIE MYÉLOGÈNE AIGUË AVANT ET APRÈS LA CHIMIOTHÉRAPIE

Il est démontré qu'une réactivité sérique cytotoxique n'exigeant pas la présence de complément apparaît dans le sérum de malades atteints de leucémie myélogène aiguë. La réaction est détectée lorsque les sérums sont incubés à court terme *in vitro* avec des globules blancs mononucléaires autochtones du sang veineux périphérique de patients au stade aigu de la maladie. Cette réactivité a été mise en évidence chez 18/18 patients. En général, la cytotoxicité était faible chez les patients au stade aigu, mais elle augmentait après la chimiothérapie et atteignait son niveau maximum au début de la rémission clinique ou juste avant. Elle n'a pu être mise en évidence contre les globules blancs autochtones de sujets en période de rémission. L'activité sérique pouvait être absorbée et éluée sur colonne contenant une préparation de protéine A et de Sepharose CL-4B; on la retrouvait dans la fraction 7S des sérums après filtration sur colonne de Sephadex G-200 et chromatographie par échange d'ions, ce qui indique que la cytotoxicité observée est due à des immunoglobulines de la classe IgG. Les auteurs pensent que des cellules porteuses de récepteurs Fc présentes dans les préparations de cellules cibles jouent le rôle de cellules effectrices. La réaction est appelée cytotoxicité cellulaire associée à l'anticorps (AACC).

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POSSIBLY SPECIFIC IMMUNE COMPLEXES IN SERA OF PATIENTS WITH UNTREATED ACUTE MYELOGENOUS LEUKEMIA

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Eleven patients with acute myelogenous leukemia (AML) in the acute stage of the disease were studied. Sera of seven patients were assayed for complement-dependent cytotoxicity to autochthonous AML cells and sera of four patients were tested with allogeneic target cells before and after ultrafiltration at low pH. No specific cytotoxic activity could be detected in any untreated sera. Ultrafiltration at low pH led, however, to the appearance of a significant cytotoxic activity in all of seven autochthonous and in three of four allogeneic combinations. Identical treatment of control AB sera did not result in any similar activity. The results show that potentially cytotoxic antibodies to leukemia-associated antigens are present in the acute stage of acute myelogenous leukemia. However, they do not express complement-dependent cytotoxicity in untreated sera. After ultrafiltration at low pH significant complement-dependent cytotoxicity was detectable in most of the sera. One possible explanation is that cytotoxic antibodies to leukemia-associated antigens are present in the form of circulating immune complexes. Splitting of them at low pH and elimination of the antigen component might result in an increased amount of free cytotoxic antibodies.

Several studies suggest the existence of leukemia-associated antigens in human acute leukemia (Harris *et al.*, 1971; Metzgar *et al.*, 1972). We have demonstrated specific cytotoxic antibodies against autochthonous leukemia cells in patients with acute myelogenous leukemia (AML). Using complement-dependent cytotoxicity we demonstrated specific antibodies in 8/8 cases (Fäldt and Ankerst, 1977). Antibody associated cellular cytotoxicity (AACC) was demonstrated in 18/18 patients after chemotherapy (Fäldt and Ankerst, 1979). With both techniques the antibodies reached the highest level of cytotoxicity just before clinical remission or in the early remission stage. Later in remission and in the relapse stage of the disease the specific cytotoxicity decreased. After successful chemotherapy in the relapse stage AACC-activity again became detectable.

Circulating immune complexes have been demonstrated in patients with acute and chronic myeloid and non-myeloid leukemias (Carpentier *et al.*, 1977) but the significance and the specificity of these have been questioned (Claque *et al.*, 1978).

A method for dissociation of antibodies from antigen-antibody complexes by ultrafiltration at low pH and demonstration of the free antibodies has been described by Sjögren *et al.* (1971) in a mouse tumor system. In the present investigation we have used this technique followed by complement-dependent cytotoxicity assays. An increase of specific cytotoxicity to autochthonous leukemia cells after ultrafiltration of sera at low pH may indicate that specific antibodies are present in the form of circulating antigen-antibody complexes which are dissociated resulting in an increased amount of free antibodies.

MATERIAL AND METHODS

Patients and sera

Eleven patients with acute myelogenous leukemia were studied. Some important clinical and laboratory data are recorded in Table 1. Peripheral white blood cells from the acute stage of the disease were obtained before therapy was initiated and sequential sera were harvested from the patients before therapy and in the course of the disease during periods without drug administration.

Normal sera from healthy AB blood group donors and sera from patients with acute myelogenous leukemia were stored at –20°C or at –80°C. They were inactivated at 56°C for 30 min before use.

Complement

Rabbit sera or equal amounts of rabbit serum and serum from a healthy AB blood group donor were used as the source of complement at the dilutions 1/4-1/8. For each patient's leukemia cells a suitable rabbit was selected as complement donor. Only a few rabbit sera were found to be suitable as the source of complement for target cells from a given donor. Complement inactivation was performed by heating for 30 min at 56°C.

Ultrafiltration of sera at low pH

Before ultrafiltration the sera were heat-inactivated. Ultrafiltration chambers of 200 ml volume and Amicon XM-100 membranes (Amicon Corporation, Lexington, Mass., USA) were used. A volume of 50 ml of 0.1 M glycine-saline buffer, pH 3.1, were added to 5 ml of serum and ultrafiltration continued until the volume of the retentate had decreased to 5 ml. This procedure was repeated twice. Then the retentates were washed thrice with 50 ml PBS, pH 7.2, and concentrated to the original serum volume.

Preparation of target cells

Autochthonous and allogeneic peripheral white blood cells from patients with acute myelogenous leukemia in the acute stage of the disease were used in the experiments as previously described (Fäldt and Ankerst, 1977). The cells were frozen and stored in liquid nitrogen and after thawing they were used in the experiments.

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TABLE I - CLINICAL AND LABORATORY DATA ON FOURTEEN INVESTIGATED PATIENTS WITH ACUTE MYELOGENOUS LEUKEMIA AT THE TIME OF SERUM COLLECTION

Pat. No., sex and age (years)	Serum[1]	Number of peripheral white blood cells × 10^{-3} per μl and % of blast cells		% of blast cells in the bone marrow	Clinical stage of the disease	Chemotherapy[4]
		WBC	Blasts			
2 Female, 27	PA-2 PF-2	62.8 24.4	90% 54%	95% ND	Before treatment After 4 weeks of treatment without remission	O × 1 + prednimustine daily
4 Male, 39	PA-4 PC-4	35.4 0.7	59% ND[2]	92% Aplasia[3]	Before treatment After 6 weeks of treatment without remission	TRAP × 2
10 Female, 59	PA-10 PD-10	58.5 1.5	40% ND	73% 9%	Before treatment After 16 weeks of treatment without remission	TRAP × 4
16 Male, 39	PA-16 PD-16	13.2 4.7	43% 0%	98% < 5%	Before treatment In complete remission after 12 weeks of treatment	O × 3 prednimustine daily
29 Male, 50	PA-29 PG-29	88.0 4.0	47% 0%	83% < 5%	Before treatment In complete remission after 18 weeks of treatment	TRAP × 4 + COAP × 4
37 Female, 32	—	6.4	7%	86%	Before treatment	—
40 Male, 72	PA-40	3.0	80%	ND	Before treatment	—
45 Female, 16	PA-45	96.0	81%	91%	Before treatment	—
46 Female, 44	PA-46	0.6	19%	95%	Before treatment	—
50 Female, 30	—	75.0	ND	70%	Before treatment	—
51 Female, 54	—	23.5	60%	35%	Before treatment	—
58 Male, 47	PA-58	4.8	7%	>50%	Before treatment	—
59 Male, 64	PA-59 PB-59	160.0 1.5	92% 0%	90% 60%	Before treatment After 4 weeks of treatment without remission	— TRAP × 2
60 Female, 39	PA-60	7.2	8%	93%	Before treatment	—

Sera were designated: PA as patients' sera taken before any treatment, PB-PG as patients' sera taken at various stages of the disease during chemotherapy. The letters are followed by the patients' number. [2]Not done. [3]Lack of bone marrow cells as a consequence of chemotherapy. [4]The following abbreviations are used: O = vincristine, prednimustine = chlorambucil-prednisolone, TRAP = thioguanine-daunorubicin-cytarabine-prednisolone, COAP = cyclophosphamide-vincristine-cytarabine-prednisolone.

TABLE II INCREASE OF SPECIFIC COMPLEMENT-DEPENDENT CYTOTOXICITY OF AUTOCHTHONOUS SERA AGAINST WHITE BLOOD CELLS OF PATIENTS WITH ACUTE MYELOGENOUS LEUKEMIA BEFORE AND AFTER ULTRAFILTRATION AT LOW pH

Experiment	Serum[1]	Complement[2]	% complement-dependent						Increase of cytotoxicity after treatment
			% ^{51}Cr-release ± SE		^{51}Cr-release ± SF		Difference to control serum[3]		
			Before treatment	After treatment	Before treatment	After treatment	Before treatment	After treatment	
1. Pat. 2	AB-2	HKC-14 1:6±	39.5±3.0 43.9±4.2	39.5±1.4 35.2±2.1	–4.4±3.0	4.3±1.4	—	—	8.7 p>0.05
	PA-2	HKC-14 1:6±	43.3±1.3 40.1±1.7	60.4±2.1 34.0±0.2	3.2±1.3	26.4±2.1	7.6 p>0.05	22.1 p<0.01	23.2 p<0.01
	PF-2	HKC-14 1:6±	50.6±4.2 49.9±4.2	—	0.7±4.2	—	5.1 p>0.05	—	—
2. Pat. 4	Ab-3	HKC-14 1:6±	32.3±2.0 27.8±0.3	33.5±0.5 28.6±0.2	4.5±2.0	4.9±0.5	—	—	0.4 p>0.05
	PA-4	HKC-14 1:6±	35.6±0.7 35.9±0.2	89.1±2.8 36.5±1.5	–0.3±0.7	52.6±2.8	–4.8 p>0.05	47.7 p<0.01	52.9 p<0.01
	PC-4	HKC-14 1:6±	35.8±0.3 23.3±0.2	—	12.5±0.3	—	8.0 p<0.05	—	—
3. Pat. 10	AB-5	KC-43 1:4±	24.1±1.9 27.2±0.9	15.1±0.6 15.4±0.3	–3.1±1.9	–0.3±0.6	—	—	2.8 p>0.05
	PA-10	KC-43 1:4±	15.8±0.9 17.6±1.1	28.6±0.8 20.4±1.6	–1.8±0.9	8.2±0.8	1.3 p>0.05	8.5 p<0.01	10.0 p<0.01
	PD-10	KC-43 1:4±	17.9±2.1 22.0±0.9	—	–4.1±2.1	—	–1.0 p>0.05	—	—
4. Pat. 16	AB-3	KC-43 1:4±	30.2±0.9 29.9±0.5	20.6±0.9 20.6±0.6	0.3±0.9	0.0±0.9	—	—	–0.3 p>0.05
	PA-16	KC-43 1:4±	32.6±0.2 29.8±2.6	31.0±0.5 24.9±0.3	2.8±0.2	6.1±0.5	2.5 p>0.05	6.1 p<0.05	3.3 p<0.05
	PD-16	KC-43 1:4±	32.2±0.9 26.7±0.5	—	6.1±0.9	—	5.8 p<0.05	—	—
5. Pat. 29	AB-10	KC-83 1:6±	38.4±0.7 38.3±1.3	25.2±0.7 23.9±0.0	0.1±0.7	1.3±0.7	—	—	1.2 p>0.05
	PA-29	KC-83 1:6±	45.5±0.9 46.6±0.0	30.4±0.1 24.0±0.9	–1.1±0.9	6.4±0.1	–1.2 p>0.05	5.1 p<0.01	7.5 p<0.01
	PG-29	KC-83 1:6±	40.2±1.0 36.0±0.2	—	4.2±1.0	—	4.1 p<0.05	—	—
6. Pat. 45	AB-20	KC-154 1:6±	13.0±2.0 6.9±0.4	16.2±0.4 8.3±0.0	6.1±2.0	7.9±0.4	—	—	1.8 p>0.05
	PA-45	KC-154 1:6±	8.3±0.3 7.0±0.3	23.3±1.4 8.2±0.5	1.3±0.3	15.1±1.4	–4.8 p>0.05	7.2 p<0.05	13.8 p<0.01
7. Pat. 59	AB-25	KC-151 1:4±	8.5±0.3 8.2±0.3	8.4±0.1 7.3±0.4	0.3±0.3	1.1±0.1	—	—	0.8 p>0.05
	PA-59	KC-151 1:4±	10.3±0.6 8.6±0.1	12.0±0.3 7.2±0.1	1.7±0.6	4.8±0.3	1.4 p>0.05	3.7 p<0.01	3.1 p<0.05
	PB-59	KC-151 1:4±	12.4±0.6 7.9±0.6	—	4.5±0.6	—	4.2 p<0.05	—	—

[1]See Footnote 1 Table I. – [2]Rabbit sera KC-14 154 or mixtures of equal amounts of rabbit and human serum, HKC-14, were used as unheated (+) and heated to 56°C for 30 min (–), respectively, and diluted to the denoted dilution. – [3]The probability that the recorded difference is due to chance was calculated by the Student's t-test.

In vitro assay of complement-dependent cytotoxicity

Autochthonous and allogeneic leukemia cells from patients with acute myelogenous leukemia in the acute stage of the disease before initiation of clinical therapy were used as target cells after storage in liquid nitrogen. After thawing in a 40°C water bath the cell suspensions were washed twice in PBS with 10% fetal calf serum and 2% Hepes at 37°C and room temperature (20-24°C), respectively. Appropriate labelling procedure with ^{51}Cr in the form of sodium chromate (New England Nuclear, Boston, Mass., USA, spec. act. 200-500 Ci/g) varied from patient to patient. The incubation time with ^{51}Cr was either 3-6 h or 15-17 h at 37°C. The alternative giving optimal sensitivity in testing was chosen for each patient.

The 3 h incubation was performed at 37°C with 1.5×10^6 viable cells suspended in 2 ml PBS with 5% fetal calf serum, 1% Hepes and 0.5 mCi/ml ^{51}Cr. After the incubation the fluid was removed and 1 ml of fetal calf serum containing 2 drops (50 μl) Varidase (Lederle Laboratories, Wayne, N.J., USA) (10.000 IU/ml) was added to the cells. After about 30 sec, 10 ml PBS with 10% fetal calf serum and 2% Hepes were added and the cell suspensions left at 4°C for 21-22 h. Before testing the supernatant was replaced with 5 ml PBS with 10% fetal calf serum and 2% Hepes. After addition of 0.5 ml Varidase the cell suspensions were left at 37°C in a water bath for 15-30 min. Five ml of cold (4°C) PBS with 10% fetal calf serum and 2% Hepes were added and the cell suspensions spun at 150 G for 5 min. The cells were suspended in PBS with 10% fetal calf serum and 1 mM $MgCl_2$, not containing $CaCl_2$, and 2% Hepes. After 15 min at 4°C aliquots of 1.0 ml cell suspension containing 10^5 viable cells were spun at 400 *g* for 1 min, and sedimented cells resuspended in test serum of the desired dilution. All experiments were performed in duplicate sets. A final concentration of 2.5×10^4 cells/0.1 ml of test serum was incubated for 15 min at room temperature (20-24°C). During the incubation the cells were occasionally shaken. Next, 0.5 ml of rabbit complement or equal amounts of human and rabbit complement diluted in PBS with 10% fetal calf serum and lmM $MgCl_2$, not containing $CaCl_2$, and 2% Hepes was added and the cell suspensions were incubated for another 15 min at room temperature.

After addition of 4 ml PBS with 10% fetal calf serum and 1 mM $MgCl_2$, not containing $CaCl_2$, and 2% Hepes cooled to 4°C the cells were centrifugated at 700 *g* for 5 min. After 45 min at 4°C 1 ml of the supernatant and the original tubes were counted in a gamma counter.

Alternatively the incubation was prolonged for 15-17 h at 37°C and the cells were suspended in Waymouth's medium with 15% fetal calf serum, 1% Hepes and 0.5 mCi/ml ^{51}Cr. After the incubation the cells were washed twice in PBS with 10% fetal calf serum and 2% Hepes and then the cells were suspended in the same fluid containing 10^5 viable cells/ml. The cell suspensions were then handled in the same way as in the tests described above with the exception that 4 ml of phosphate buffered saline with 0.02% EDTA were added after the incubation with complement. The percentage of isotope release was calculated as follows:

$$\frac{\text{Radioactivity in 1.0 ml of supernatant} \times 4.6 \times 100}{\text{Total radioactivity/tube}}$$

The statistical significance of the recorded differences between complement-dependent effects of test and control sera was calculated by Student's t-test.

RESULTS

Sera from 11 patients and leukemia cells from 10 patients with acute myelogenous leukemia were studied and the results are summarized in Tables I-III. Data summarizing important laboratory and clinical parameters of the disease at the time of collection of sera and leukemia cells are presented in Table I. Sera from patients with leukemia and from healthy AB blood group donors were tested untreated and after ultrafiltration at low pH for complement-dependent cytotoxicity against autochthonous and allogeneic leukemia cells (Table II). After ultrafiltration of the same sera at low pH they demonstrated significant cytotoxic activity against the same target cells and they also showed significant increase of cytotoxicity as compared to autochthonous untreated sera. Reference control AB sera did not show any significant increase of cytotoxicity after identical treatment. Autochthonous leukemia sera obtained after chemotherapy demonstrated significant specific complement-dependent cytotoxicity in 4/6 tested patients. Five allogeneic leukemia sera were tested for complement-dependent cytotoxicity against target cells from 4 different patients with acute myelogenous leukemia (Table III). None of five sera from patients with untreated leukemia showed significant cytotoxicity as compared to allogeneic reference control AB sera, when tested on allogeneic leukemia target cells. After ultrafiltration at low pH, four leukemia sera demonstrated significant cytotoxicity as compared to the control sera and three of them showed significant increase of cytotoxicity as compared to untreated serum.

DISCUSSION

The appearance of circulating immune complexes in acute and chronic myeloid and non-myeloid human leukemias has been demonstrated using the ^{125}I-Clq-binding test (Carpentier *et al.*, 1977). An increased serum ^{125}I-Clq-binding was demonstrated in 40% of patients with acute myelogenous leukemia and a negative correlation to the clinical course of the disease was reported. Most patients who lacked detectable immune complexes entered remission, while the patients with detectable immune complexes did not. The specificity of the detected immune complexes can not be demonstrated with this technique and the significance of the results has been questioned (Claque *et al.*, 1978) on the basis of a demonstrated strong correlation between presence of immune complexes and a history of recent infection and lack of correlation to the prognosis of the leukemic disease.

TABLE III

INCREASE OF SPECIFIC COMPLEMENT-DEPENDENT CYTOTOXICITY OF SERA FROM PATIENTS WITH ACUTE MYELOGENOUS LEUKEMIA AGAINST ALLOGENEIC LEUKEMIC WHITE BLOOD CELLS BEFORE AND AFTER ULTRAFILTRATION AT LOW pH

Target-cell donor	Serum[1]	Complement[2]	% complement dependent						Increase of cytotoxicity after treatment
			% ^{51}Cr-release ± SE		^{51}Cr-release ± SE		Difference to control serum[3]		
			Before treatment	After treatment	Before treatment	After treatment	Before treatment	After treatment	
No. 37	AB-27	KC-166 1:4±	10.0±0.6 10.9±1.1	6.3±0.3 5.6±0.6	–0.9±0.6	0.7±0.3	—	—	1.6 p>0.05
	PA-40	KC-166 1:4±	12.2±0.7 11.5±0.6	11.5±1.3 6.7±0.7	0.7±0.7	4.8±1.3	1.6 p>0.05	4.1 p<0.05	4.1 p>0.05
No. 45	AB-26	KC-154 1:6±	8.6±0.5 6.9±0.1	6.8±1.1 3.8±0.0	1.7±0.5	0.3±1.1	—	—	1.3 p>0.05
	PA-59	KC-154 1:6±	9.3±0.4 7.0±0.3	8.9±0.4 5.4±0.3	2.3±0.4	3.5±0.4	0.6 p>0.05	0.5 p>0.05	1.2 p>0.05
	PA-60	KC-154 1:6±	10.3±0.1 7.5±0.0	16.0±0.6 4.6±0.2	2.8±0.1	11.4±0.6	1.1 p>0.05	8.4 p<0.05	8.6 p<0.01
No. 50	AB-26	KC-151 1:8±	3.2±0.2 2.5±0.1	2.1±0.2 1.4±0.4	0.7±0.2	0.7±0.2	—	—	0.0 p>0.05
	PA-58	KC-151 1:8±	3.5±0.4 3.7±0.1	15.9±1.4 2.6±1.1	–0.2±0.4	13.3±1.4	–0.9 p>0.05	12.6 p<0.01	13.5 p<0.01
No. 51	AB-24	KC-151 1:4±	15.1±0.3 14.2±0.4	10.0±0.9 9.3±0.1	0.9±0.3	0.7±0.9	—	—	–0.2 p>0.05
	PA-46	KC-151 1:4±	15.4±0.8 14.9±0.3	18.1±1.9 8.9±0.2	0.5±0.8	9.2±1.9	–0.4 p>0.05	8.5 p<0.05	8.7 p<0.05

[1] See Footnote 1 Table I. - [2] Rabbit sera KC-151 – 166 were used as unheated (+) and heated to 56°C for 30 min (-), respectively, and diluted to the denoted dilution. – [3] The probability that the recorded difference is due to chance was calculated by the Student's t-test.

In the present investigation no significant specific complement-dependent cytotoxicity against autochthonous leukemia cells could be detected in the untreated sera from seven patients with acute myelogenous leukemia harvested at the acute stage of the disease before initiation of any therapy. This is in accordance with our previously reported data (Fäldt and Ankerst, 1977). Specific complement-dependent cytotoxicity against the same target cells became demonstrable in all these sera after ultrafiltration at low pH. The increase in activity was statistically significant for most sera. Reference control AB sera did not show any similar increase of complement-dependent cytotoxicity after identical treatment. Tests for complement-dependent cytotoxicity on allogeneic leukemic target cells gave analogous results. None of the untreated sera exerted significant cytotoxic effect but ultrafiltration of the sera at low pH resulted in significant cytotoxic activity in most sera tested.

Sera obtained from the leukemia patients after chemotherapy demonstrated significant specific complement-dependent cytotoxicity on autochthonous leukemia target cells in 4/6 tested patients, which is in accordance with our previous results (Fäldt and Ankerst, 1977).

The presented results indicate that potentially cytotoxic antibodies to leukemia-associated surface antigens are present in the sera of patients with untreated acute myelogenous leukemia. However, they are often not detectable unless the sera are ultrafiltrated at low pH. At least two possible explanations are to be considered. The cytotoxic antibodies may be bound to postulated soluble leukemia antigens in the form of circulating immune complexes. Splitting of them at low pH and elimination of the antigen component might result in an increased amount of free cytotoxic antibodies detectable in complement-mediated cytotoxicity assays. The presence of immune complexes in acute myelogenous leukemia has been shown but the specificity has not been demonstrated (Carpentier *et al.*, 1977; Claque *et al.*, 1978). Using a method described by Sjögren *et al.* (1971) for dissociation of antigen-antibody complexes by ultrafiltration at low pH, we demonstrate specific complement-dependent cytotoxicity to autochthonous leukemia cells in the retentates of most of the ultrafiltrated sera. This procedure should result in an increase of the amount of free antibody, provided the postulated leukemia antigen has a MW less than 100,000. Another alternative is that interfering non cytotoxic antibodies may prevent the cytotoxic antibodies to express specific cytotoxicity by competition at the antigen sites or the latter antibodies may be blocked by anti-idiotypic antibodies. If the interfering non cytotoxic antibodies or anti-idiotypic antibodies are sensitive to acid treatment, they may be destroyed at pH 3.1, leaving any acid resistant cytotoxic leukemia antibodies free to express specific cytotoxicity. However, the present knowledge about the sensitivity of immunoglobulins to pH 3.1 makes this possibility less probable.

The analogous results or tests against allogeneic myelogenous leukemia target cells indicate that at least some of the leukemia-associated antigens are cross-reacting or are shared by several of the acute myelogenous leukemias. However, this point remains to be further clarified before more definite conclusions may be drawn.

The present investigation and the fact that specific cytotoxic antibodies were detected in all patients with this form of leukemia after successful chemotherapy (Fäldt and Ankerst, 1977; Fäldt and Ankerst, 1979) may indicate that specific antigen-antibody complexes are frequently present at the acute stage of acute myelogenous leukemia before therapy. However, the proportion of leukemia sera containing such complexes can not be estimated from this study, since we have not directly demonstrated the presence of complexes in the sera and since the patients were selected, most of them with pronounced leukocytosis.

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A ^{125}I PROTEIN A BINDING ASSAY DETECTING CELL SURFACE ANTIGENS: EVIDENCE FOR THE PRESENCE OF SPECIFIC ANTIBODIES AGAINST LEUKEMIA-ASSOCIATED ANTIGENS IN HUMAN LEUKEMIAS.

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SUMMARY

A ^{125}I protein A binding assay detecting antibodies to cell surface antigens on human blood cells was developed and evaluated using sera from multitransfused nonleukemic patients sensitized against HLA antigens. The binding assay was found to be reproducible and more sensitive than conventional HLA testing. Seven patients with acute myelogenous leukemia and two patients with acute lymphoblastic leukemia successfully treated by chemotherapy were investigated. Seven of nine patients' sera obtained in partial or complete remission showed significant binding to autochthonous leukemic bone marrow cells or peripheral white blood cells. In two cases sera from the acute stage of the disease demonstrated the largest binding to the patients own leukemic cells. Sera of four patients with demonstrable significant binding to autochthonous leukemic target cells failed to bind to autochthonous remission cells when both types of target cells were tested in parallel. It was found that differences in serum concentrations of IgG, IgA and IgM could not explain the demonstrated increased binding of autochthonous sera to leukemic target cells as compared to control sera. We propose that the presented ^{125}I protein A binding assay detects antibodies selectively reactive with acute leukemia cells.

INTRODUCTION

The reaction of Staphylococcus aureus protein A with the Fc region of most mammalian IgG molecules (12) has been widely used in the analysis of cell surface markers. Several methods for iodination of protein A with ^{125}I have been introduced (3, 5, 13). Despite several attempts there are few reports on the presence of specific antitumor antibodies in man using ^{125}I protein A binding assays. Lack of sensitivity and high background levels using undiluted sera or sera at low dilutions have been common problems.

In previous studies we have presented evidence for the presence of specific cytotoxic leukemia antibodies in adult patients with AML (6, 7). These antibodies could be detected only in undiluted sera or in sera at low dilutions and most often only after successful chemotherapy. We have also presented indirect evidence for the presence of specific immune complexes in sera of patients with untreated leukemia using ultrafiltration of the sera at low pH (8).

The aim of the present study was to develop a sensitive ^{125}I protein A binding assay allowing the detection also of non-cytotoxic antibodies to leukemic cells in patients with acute leukemia.

MATERIAL AND METHODS

Patients

Seven patients with acute myelogenous leukemia (AML) and two patients with acute lymphoblastic leukemia (ALL) successfully treated by chemotherapy were studied. Some important clinical and laboratory data are presented in Table 1. Bone marrow cells and peripheral white blood cells from the acute stage of the disease were obtained before therapy was initiated and in some cases remission bone marrow or peripheral white blood cells were harvested. Sera from the patients were obtained before therapy and then sequentially in the course of the disease. Bone marrow cells and peripheral white blood cells from healthy blood donors and normal sera from AB blood group donors were also collected.

Sera

Sera were stored at -80^{o}C or at -196^{o}C in liquid nitrogen. They were decomplemented at 56^{o}C for 30 min before use.

Target cells

Blood and bone marrow cells from patients in the acute and remission stages of the disease and cells from healthy blood donors were separated from heparinized bone marrow aspirates or whole venous blood by centrifugation on Ficoll-Isopaque (density 1.077 g/cm^{3}). The cells in the interphase were frozen and stored in liquid nitrogen and after thawing they were used in the experiments.

Iodination of protein A (^{125}I protein A)

Protein A was purchased from Pharmacia, Uppsala, Sweden. The Bolton-Hunter reagent was used for iodination with ^{125}I according to the method described by Langone et al (13).

^{125}I protein A binding assay

After thawing in a 40°C water bath the cell suspensions were washed in PBS with 3% bovine serum albumine (BSA) and 0.1% Tween-20 at 37°C and resuspended in the same medium at room temperature (20-24°C). The same solution was used for dilutions and washings throughout the tests. It was found important to use freshly prepared solutions in all tests to get reliable results. All tubes were washed 30 min at room temperature with the solution before use. Cell suspensions in a volume of 0.1 ml containing 2.5-5x10^5 viable cells were incubated for 30 min with 0.1 ml of test serum at the desired dilution at room temperature.

The cells were washed trice and then put into new tubes. After another washing 0.1 ml of ^{125}I protein A (corresponding to 10000-15000 CPM) was added. After 30 min at room temperature followed by four washings the cells were collected and put into new tubes, which were measured in a gamma counter.

All samples were tested in two or three parallel tubes. Each set of tests included tubes containing cells and control tubes without cells tested with all reagents identically as the tubes containing cells. The radioactivity per sample was expressed as the difference between the tube containing cells and the corresponding tube without cells. Bound radioactivities in counts per minute (CPM) with test sera and control sera were respectively measured and the quotient between them calculated.

The statistical significance of the recorded differences in cellular binding of radioactivity with test sera as compared to control sera was calculated by Students t-test.

RESULTS

Some important clinical and laboratory data are recorded in Table 1 and the results of the experiments are summarized in Tables 2-5 and Figures 1-6. The sensitivity of the binding assay was studied by analysis of a rabbit antiserum to human leukocytes and sera of non-leukemic patients sensitized against HLA antigens. A titration of the rabbit antiserum against target mononuclear blood cells of a healthy blood donor (NL-25) is presented in Figure 1. The antibodies are detectable at dilution 1:1000.

Sera of 5 multitransfused nonleukemic patients (MT-1 - MT-5) were tested against mononuclear target cells from peripheral blood of a healthy blood donor (NL-9) in parallel with 2 reference control AB sera (AB-29, AB-31). Only 2 patients' sera (MT-1, MT-5) demonstrated significant binding to the target cells as compared to that of control sera, most probably due to HLA-sensitization (Figure 2).

When serum MT-5 was titrated against the same target cells (NL-9) it showed significant binding as compared to a control serum (AB-29) at the dilutions 1:1, 1:5 and 1:25 (Figure 3). When the same sera were tested against the same target cells using the conventional HLA testing technique (11), only undiluted MT-5 showed significant binding. In another variant of HLA testing with prolonged incubation for 24h at $37^{o}C$ (16), however, the serum demonstrated significant binding to separated B lymphocytes at the dilution 1:5.

The influence of the number of target cells on the sensitivity of the test is illustrated in Figure 4. It is concluded that the use of $2.5x10^{5}$ viable cells is optimal under these experimental conditions.

In order to test the reproducibility of the binding assay a serum from a healthy AB blood donor (AB-31) and a serum from a multitranfused patient (MT-5D) were tested repeatedly against leukemic target cells from pat.no. 79 (Table 2). Highly reproducible results were obtained with both sera and the significant increased binding of MT-5D as compared to AB-31 could be demonstrated repeatedly.

The assay was used to analyze the binding of sequential sera from 7 patients with AML (patients 28, 37, 46, 49, 60, 63 and 81) and 2 patients with ALL (patients BL-1 and 79) to autochthonous leukemic target cells (Table 3). Sera were collected from the patients at different stages of the disease as recorded in Table 1. In order to check 3 selected reference control AB sera (AB-24, AB-29 and AB-31) they were tested against allogeneic leukemic blood cells and normal mononuclear blood cells in parallel with 10 other AB sera (AB-32 - AB-41) and autochthonous serum from the latter target cell donor. The quotient between the binding of ^{125}I protein A with each of these sera and the binding with one of the reference control AB sera (AB-29) was calculated (Figure 5). It could be demonstrated that the binding of reference control AB sera to both types of target cells was closely similar to that of the other 10 AB sera and the autochthonous serum. The largest quotient between the binding of the various AB sera and serum AB-29 to both types of target cells was 1.7 and 1.6 respectively.

Significant binding to autochthonous leukemic target cells could be detected in sera of most of the patients. Usually the reactivity appeared in sera of the patients in partial or complete remission after successful chemotherapy, but in 2 cases (patients 28 and 63) the reactivity was most pronounced in sera taken before initiation of chemotherapy. Sera from two patients with ALL (BL-1A and PC-79) and one patient with AML (PF-37), were titrated against autochthonous leukemic target cells in parallel with a control serum (AB-29). Two sera showed significant binding undiluted and at dilution 1:5 (BL-1A, PC-79) as compared to the control serum. Serum PF-37 showed significant binding only when tested undiluted (Figure 6).

The concentrations of IgG, IgA and IgM were determined in all sera. Sera PB-28 and PA-63 were shown to contain elevated concentrations of IgG and IgA. It may therefore be argued that nonspecific binding of immunoglobulin to the cells may contribute to the recorded cellular uptake in these 2 cases. However, this possibility is not supported by the finding that serum PA-63 demonstrated significant binding only to autochthonous leukemic bone marrow cells, while no increased binding was found to peripheral white blood

cells in the acute or remission stages of the disease (Table 4). Also sera BL-1A, PF-37 and PB-81 lacked significant binding to peripheral white blood cells or bone marrow cells from the remission stage (Table 4). Serum PC-79 bound significantly to leukemic autochthonous peripheral white blood cells but also to allogeneic cells from one healthy donor (NL-22) but not from another (NB-1) (Table 5). None of the other tested sequential sera from leukemic patients showed significant binding to leukemic or normal allogeneic peripheral white blood cells or to allogeneic bone marrow cells from a healthy blood donor. Serum MT-5 from a multitransfused patient showed significant binding to cells of two of three tested donors (Table 5).

TABLE 1 Clinical and laboratory data on 9 patients with acute leukemia at the time of serum collection.

Pat.No. sex and age (years)	Serum[1]	Number of peripheral white blood cells per uL and % of blast cells		% of blast cells in the bone marrow	Clinical stage of the disease	Chemotherapy[2]
		WBC $x10^{-3}$	Blasts %			
BL-1	PA-1	6.9	46	92	Before treatment	
Male, 5	PC-1	4.6	0	ND[3]	After 3 weeks of treatment	Ox3+Adr x1+P+MTXx2 i.v.
	PG-1	14.4	0	ND	In complete remission after 10 weeks of treatment	+Ox3+Adr x2+P+MTXx3 +MTXx1 i.v.
28	PA-28	20.0	35	85	Before treatment	
Male, 37	PB-28	2.8	0	ND	After 2 weeks of treatment	TOMPx1
	PC-28	5.1	0	< 5	In complete remission after 34 weeks of treatment	+TOMPx1+POMPx1+COAx5
	PD-28	6.2	0	< 5	In complete remission after 62 weeks of treatment	+RAx1+COAx1
	PE-28	22.7	11	12	In relapse after 72 weeks of treatment	+COAx1
	PF-28	6.7	0	ND	In relapse after 95 weeks of treatment	+RAx2+COAx5
37	PA-37	6.4	7	86	Before treatment	
Female, 32	PB-37	2.3	ND	ND	After 4 weeks of treatment	TRAPx3
	PC-37	5.4	0	< 5	In complete remission after 9 weeks of treatment	+TRAPx1
	PD-37	6.3	0	< 5	In complete remission after 16 weeks of treatment	+TRAPx1+COAPx2
	PE-37	ND	ND	ND	In complete remission after 20 weeks of treatment	+TRAPx1
	PF-37	4.8	0	< 5	In complete remission after 26 weeks of treatment	+TRAPx1+COAPx1
46	PA-46	0.2	19	95	Before treatment	
Female, 44	PA-46	12.1	0	< 5	In complete remission after 12 weeks of treatment	TRAPx4
49	PA-49	85.0	85	93	Before treatment	
Female, 23	PB-49	6.9	3	9	After 3 weeks of treatment	RAx1
	PC-49	3.4	0	< 5	In complete remission after 6 weeks of treatment	+TRAx1
	PD-49	5.7	ND[3]	ND	In complete remission after 12 weeks of treatment	+TRAx1+TAx1
	PF-49	6.7	0	< 5	In complete remission after 26 weeks of treatment	+TRAx1+TAx1
60	PA-60	7.2	8	93	Before treatment	
Female, 39	PB-60	1.5	0	ND	After 2 weeks of treatment	TRAPx1
	PC-60	0.8	ND	64	After 4 weeks of treatment	+TRAPx1
	PD-60	3.9	0	13	In partial remission after 12 weeks of treatment	+TRAPx3
	PE-60	3.6	1	68	In relapse after 16 weeks of treatment	+TRAPx1

TABLE 1 Clinical and laboratory data on 9 patients with acute leukemia at the time of serum collection. cont. 2

Pat.No. sex and age (years)	Serum[1]	Number of peripheral white blood cells per uL and % of blast cells		% of blast cells in the bone marrow	Clinical stage of the disease	Chemotherapy[2]
		WBC x10^{-3}	Blasts %			
63	PA-63	64.0	74	95	Before treatment	
Male, 39	PB-63	3.0	0	< 5	In complete remission after 6 weeks of treatment	TRAPx2
	PC-63	3.0	0	< 5	In complete remission after 10 weeks of treatment	+TRAPx1
	PD-63	3.8	0	ND[3]	In complete remission after 13 weeks of treatment	+TRAPx1
	PE-63	3.6	0	ND	In complete remission after 17 weeks of treatment	+TRAPx1
79	PA-79	19.2	4	85	Before treatment	
Male, 60	PB-79	4.2	ND	22	After 2 weeks of treatment	O+Prednimustine
	PC-79	5.3	0	12	In partial remission after 10 weeks of treatment	+Ox3+OAdrDx+MTx i.t.
	PD-79	1.6	ND	76	In relapse after 16 weeks of treatment	+TRAPx2
81	PA-81	5.8	1	54	Before treatment	
Female,	PB-81	1.5	0	< 5	After 4 weeks of treatment	TRAPx2

Footnotes to TABLE 1

1/ Sera were designated as follows: PA, patients' sera taken before any treatment. PB-PF, patients' sera taken at various stages of the disease during chemotherapy. The letters are followed by the patients' numbers.

2/ The following abbreviations are used:
O = vincristine
Adr = adriamycin
P = prednisolone
MTX = methotrexate
TOMP = thioguanine-vincristine-methotrexate-prednisolone
POMP = mercaptopurine-vincristine-methotrexate-prednisolone
COA = cyclophosphamide-vincristine-cytarabine
RA = daunorubicin-cytarabine
TRAP = thioguanine-daunorubicin-cytarabine-prednisolone
COAP = cyclophosphamide-vincristine-cytarabine-prednisolone
TRA = thioguanine-daunorubicin-cytarabine
TA = thioguanine-cytarabine
OAdrD = vincristine-adriamycin-deltison (deltison prednisone)
Prednimustine = chlorambucil - prednisolone (esterfied)
i. v. = intravenously
i. t. = intrathecally

3/ Not done

TABLE 2 Reproducibility of the ^{125}I protein A binding assay demonstrated by testing of a serum from a healthy AB blood donor (AB-31) and serum from a multitransfused patient (MT-5D) against leukemic target cells (pat.no. 79).

		Uptake of ^{125}I protein A[1] ± SE				
Date		820419	820510	820511	820513	820514
Serum:[2]	AB-31	108± 5 (10)	122± 5 (20)	100± 2 (20)	108± 4 (10)	116± 3 (10)
	MT-5D	879± 32(10)	ND[3]	ND	686± 26(10)	585± 21(10)

Footnotes to TABLE 2

1/ Uptake of ^{125}I protein A is presented in counts per minute (CPM) ± standard error (SE). The figures within parenthesis are the number of observations.

2/ Sera were designated as follows:
AB-31 = serum of healthy donor of blood group AB
MT-5D = serum from a multitransfused nonleukemic patient sensitized against HLA antigens

3/ Not done

TABLE 3 Demonstration of antibodies against autochthonous leukemic target cells in sequential sera from 9 patients with acute leukemia at different stages of the disease.

Target cells	Serum[1]	Uptake of ^{125}I protein A ± SE and p value[2]	Quotient[3]	Immunoglobulin concentration[4]		
				IgG	IgA	IgM
Pat. BL-1, bone marrow cells	AB-29 1/4	129± 4		9.2	1.7	1.1
	BL-1A 1/4	201± 4**	1.6	9.4	1.7	1.0
	BL-1C 1/4	86± 4	0.7	6.1	1.7	0.9
	BL-1G 1/4	260± 14**	2.0	7.2	1.5	2.9
Pat. 46 bone marrow cells	AB-29 1/4	105± 4				
	PC-46 1/4	203± 8**	1.9	6.7	1.3	0.6
Pat. 49, peripheral blood cells	AB-29 1/4	113± 6				
	PA-49 1/4	155± 2*	1.4	11.0	2.1	1.7
	PB-49 1/4	105± 13	0.9	13.9	3.6	1.4
	PC-49 1/4	186± 2**	1.6	11.3	2.3	1.4
	PD-49 1/4	96± 11	0.8	9.2	1.8	1.6
	PF-49 1/4	367± 17**	3.2	7.6	1.9	1.2
Pat. 63, bone marrow cells	AB-29 1/4	176± 7				
	PA-63 1/4	348± 11**	2.0	18.6	2.7	1.9
	PB-63 1/4	255± 9**	1.4	10.3	1.1	0.8
	PC-63 1/4	229± 1**	1.3	8.2	0	0.6
	PD-63 1/4	224± 13*	1.3	8.1	0.3	0.8
	PE-63 1/4	276± 14*	1.6	8.9	0.5	0.9
Pat. 60, bone marrow cells	AB-24 1/1	155± 1		7.6	0	0.8
	PA-60 1/1	231± 9**	1.5	9.5	1.0	1.3
	PB-60 1/1	262± 10**	1.7	7.1	0.9	0.9
	PC-60 1/1	229± 17*	1.5	8.2	1.1	0.9
	PD-60 1/1	330± 12**	2.1	16.5	1.3	1.5
	PE-60 1/1	278± 2***	1.8	9.4	0.9	1.1

TABLE 3 Demonstration of antibodies against autochthonous leukemic target cells cont. 1 in sequential sera from 9 patients with acute leukemia at different stages of the disease.

Target cells	Serum[1]	Uptake of ^{125}I protein A ± SE and p value [2]	Quotient[3]	Immunoglobulin concentration [4]		
				IgG	IgA	IgM
Pat. 79, peripheral blood cells	AB-24 1/1	80± 3				
	PA-79 1/1	84± 14	1.1	7.8	1.5	0.6
	PB-79 1/1	114± 7*	1.4	7.5	1.4	0.4
	PC-79 1/1	259± 9**	3.2	6.5	1.3	0.3
	PD-79 1/1	46± 5	0.6	6.5	1.3	0.3
Pat. 81, bone marrow cells	AB-29 1/1	100± 8				
	PA-81 1/1	127± 5*	1.3	10.8	0.9	1.5
	PB-81 1/1	176± 7**	1.8	12.8	1.3	1.2
Pat. 37, bone marrow cells	AB-31 1/1	93± 1		9.1	1.9	0.8
	PA-37 1/1	77± 3	0.8	8.8	1.3	0.5
	PB-37 1/1	54± 2	0.6	12.8	1.6	0.8
	PC-37 1/1	119± 11 NS[5]	1.3	8.4	1.2	0.4
	PD-37 1/1	271± 9**	2.9	7.7	0.8	0.8
	PE-37 1/1	162± 2**	1.7	7.0	0.9	0.4
	PF-37 1/1	247± 1***	2.7	7.3	0.8	0.5
Pat. 28, peripheral blood cells	AB-31 1/1	114± 10				
	PB-28 1/1	228± 2**	2.0	18.1	5.7	0.9
	PC-28 1/1	204± 2**	1.8	ND[6]	ND	ND
	PE-28 1/1	180± 5*	1.6	8.4	1.8	0.5
	PF-28 1/1	93± 7	0.8	9.4	2.2	0.5

Footnotes to TABLE 3

1/ Sera were designated as follows: AB, sera of healthy donors of blood group AB followed by the donor's number. PA, patients' sera taken before any treatment. PB-PF, patients' sera taken at various stages of the disease during chemotherapy. The letters are followed by the patients' numbers and serum dilution. PA-28 was not available for testing.

2/ Uptake of ^{125}I protein A is presented in counts per minute (CPM) ± Standard error (SE). The probability that the recorded differences between patients' sera and control sera are due to chance was calculated by Student's t-test.
* = $p < 0.05$ ** = $p < 0.01$ *** = $p < 0.0005$

3/ The quotient between the radioactivity uptake with patients' sera and control AB serum is presented.

4/ The values are expressed as g/l. Normal values: IgG: 6.5-15.0, IgA: 0.7-2.4, IgM: 0.5-2.0.

5/ Not significant

6/ Not done

TABLE 4 Comparison between uptake of ^{125}I protein A on autochthonous bone marrow and on peripheral white blood cells from 4 patients with acute leukemia at different stages of the disease.

Target cell donor	Serum [1]	Bone marrow cells from the acute stage of the disease: Uptake of ^{125}I protein A ± SE and p value [2]	Quotient [3]	Peripheral white blood cells from the acute stage of the disease: Uptake of ^{125}I protein A ± SE and p value	Quotient	Bone marrow cells from the same patient in remission: Uptake of ^{125}I protein A ± SE and p value	Quotient	Peripheral white blood cells from the same patient in remission: Uptake of ^{125}I protein A ± SE and p value	Quotient
Pat.	AB-29 1/1	62± 5		80± 10		ND [4]		63± 4	
BL-1	BL-1A 1/1	187± 11**	3.0	147± 7*	1.8	ND		60± 5 NS [5]	1.0
Pat.	AB-29 1/1	84± 4		68± 6		ND		56± 3	
37	PF-37 1/1	172± 6**	2.1	172± 9**	2.5	ND		48± 4 NS	0.8
Pat.	AB-29 1/5	153± 14		101± 7		ND		78± 8	
63	PA-63 1/5	283± 3**	1.8	130± 8 NS	1.2	ND		86± 1 NS	1.1
Pat.	AB-31 1/1	169± 12				217± 17			
81	PA-81 1/1	148± 7 NS	0.9	ND		205± 17	0.9	ND	
	PB-81 1/1	304± 11**	1.8	ND		140± 10	0.6	ND	

Footnotes to TABLE 4

1/ See Footnote 1, Table 3
2/ See Footnote 2, Table 3
3/ See Footnote 3, Table 3
4/ Not done
5/ Not significant

TABLE 5 Comparison between uptake of ^{125}I protein A on allogeneic peripheral white blood cells from 1 patient with acute myelogenous leukemia and on bone marrow cells and peripheral white blood cells from 2 healthy donors.

Serum [1]	Peripheral white blood cells from a patient with leukemia (Pat. 79)		Peripheral white blood cells from a healthy blood donor (NL-22)		Bone marrow cells from a healthy blood donor (NB-1)	
	Uptake of ^{125}I protein A $\pm$ SE and p value	Quotient [3]	Uptake of ^{125}I protein A $\pm$ SE and p value	Quotient	Uptake of ^{125}I protein A $\pm$ SE and p value	Quotient
AB-24 1/1	129 ± 12		93 ± 13		103 ± 15	
PF-37 1/1	77 ± 12	0.6	66 ± 4	0.7	36 ± 5	0.3
PF-49 1/1	64 ± 2	0.5	23 ± 2	0.2	97 ± 1	0.9
PD-60 1/1	104 ± 12	0.8	103 ± 19 NS [4]	1.1	47 ± 15	0.5
PC-79 1/1	ND [5]		479 ± 56*	5.1	106 ± 14	1.0
MT-5C 1/1	938 ± 20***	7.3	301 ± 3**	3.2	108 ± 9	1.0

Footnotes to TABLE 5

1/ Sera were designated as follows: AB-24, serum of a healthy donor of blood group AB. PC-PF, sera of 4 leukemic patients obtained after successful chemotherapy. MT-5C, serum from a multitransfused nonleukemic patient sensitized against HLA antigens.

2/ See Footnote 2, Table 3

3/ See Footnote 3, Table 3

4/ Not significant

5/ Not done

Fig.1

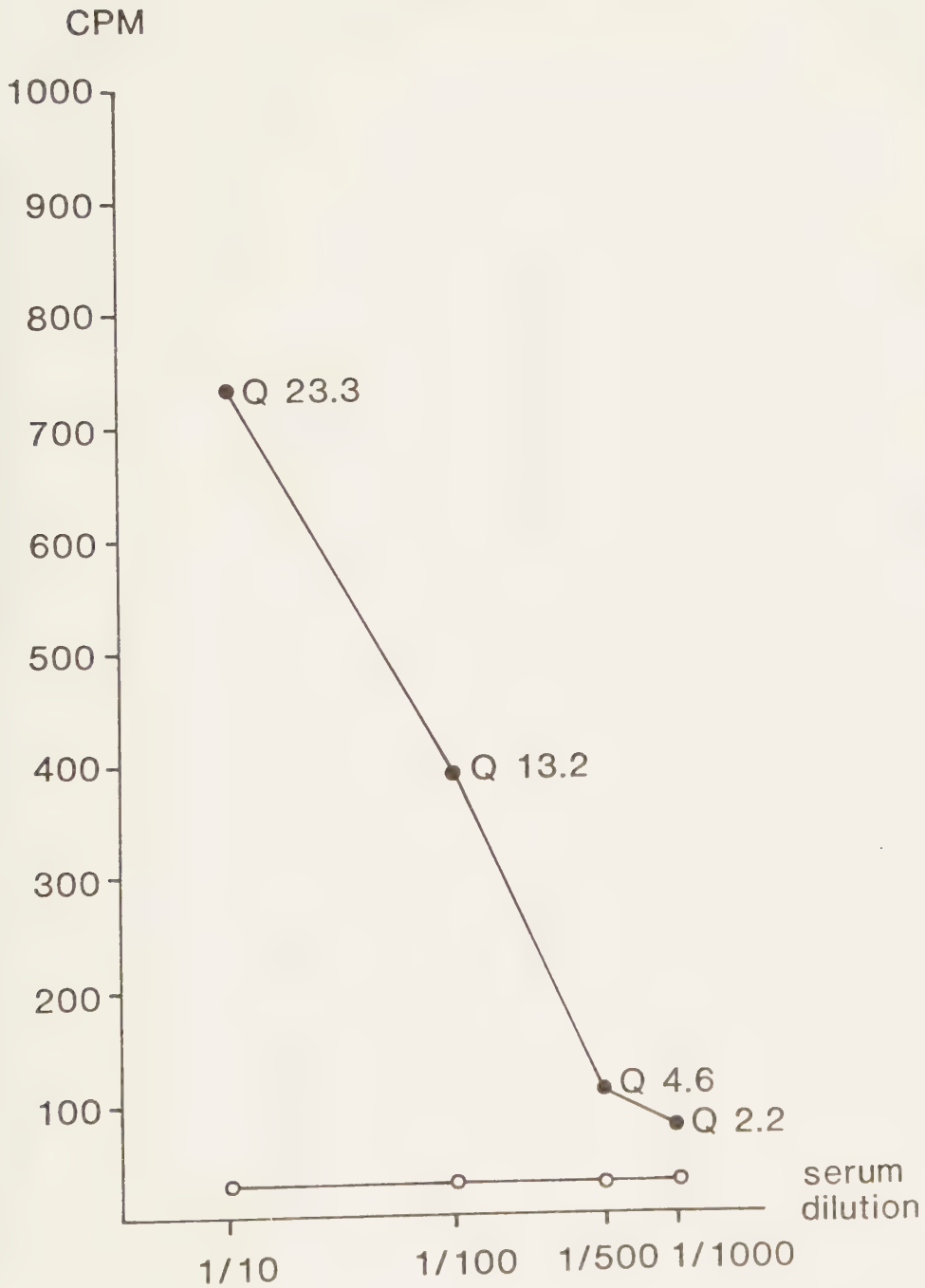

FIGURE 1 Titration of a rabbit antiserum (KC-175, ●—●) against human mononuclear white blood cells (NL-25) from a healthy blood donor as compared to the preimmune serum (KC-175, o—o).

Fig.2

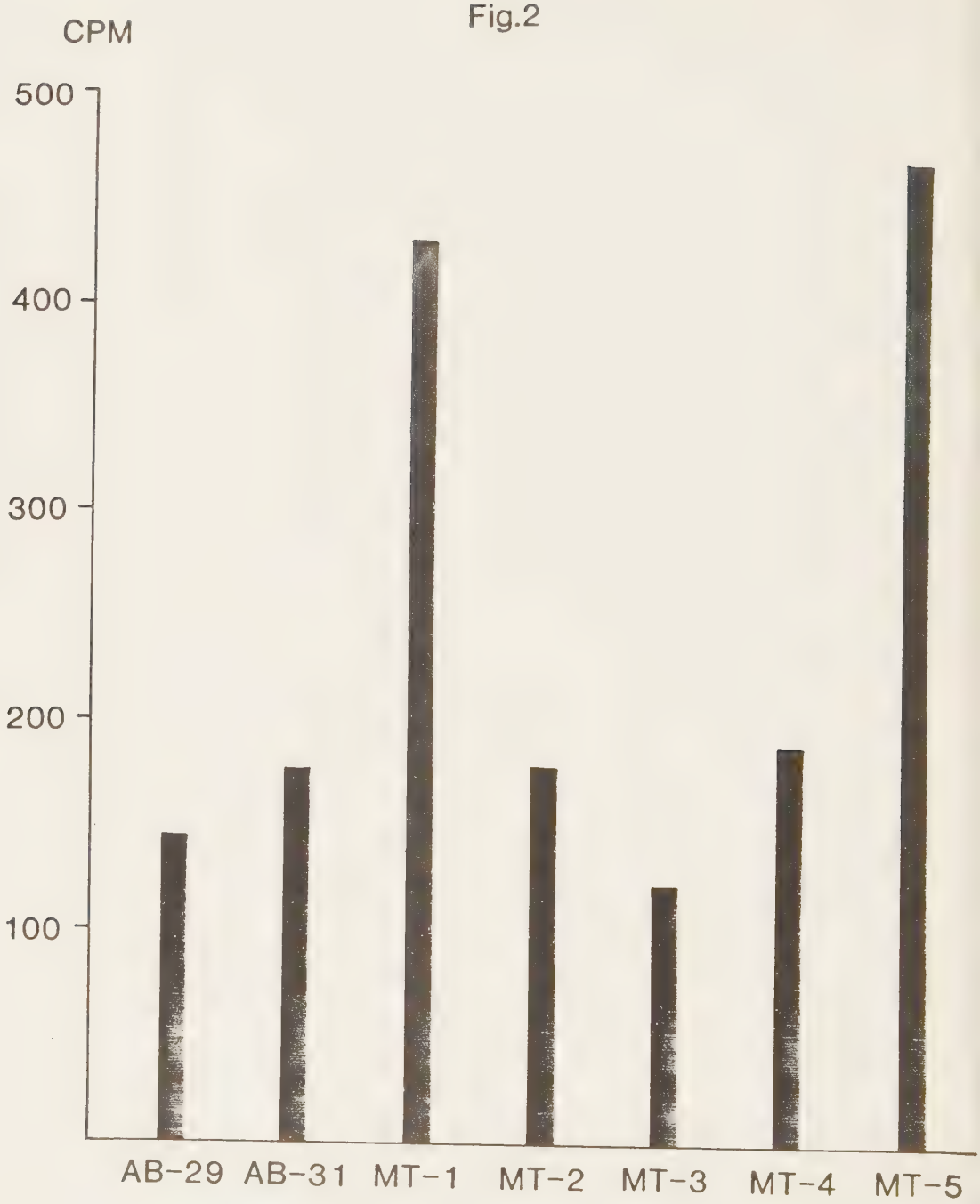

FIGURE 2 Radioactivity uptake after incubation of human mononuclear white blood cells from a healthy blood donor (NL-9) with sera from five multitransfused patients (MT 1-5) and sera from two healthy AB blood group donors (AB-29, AB-31).

Fig.3

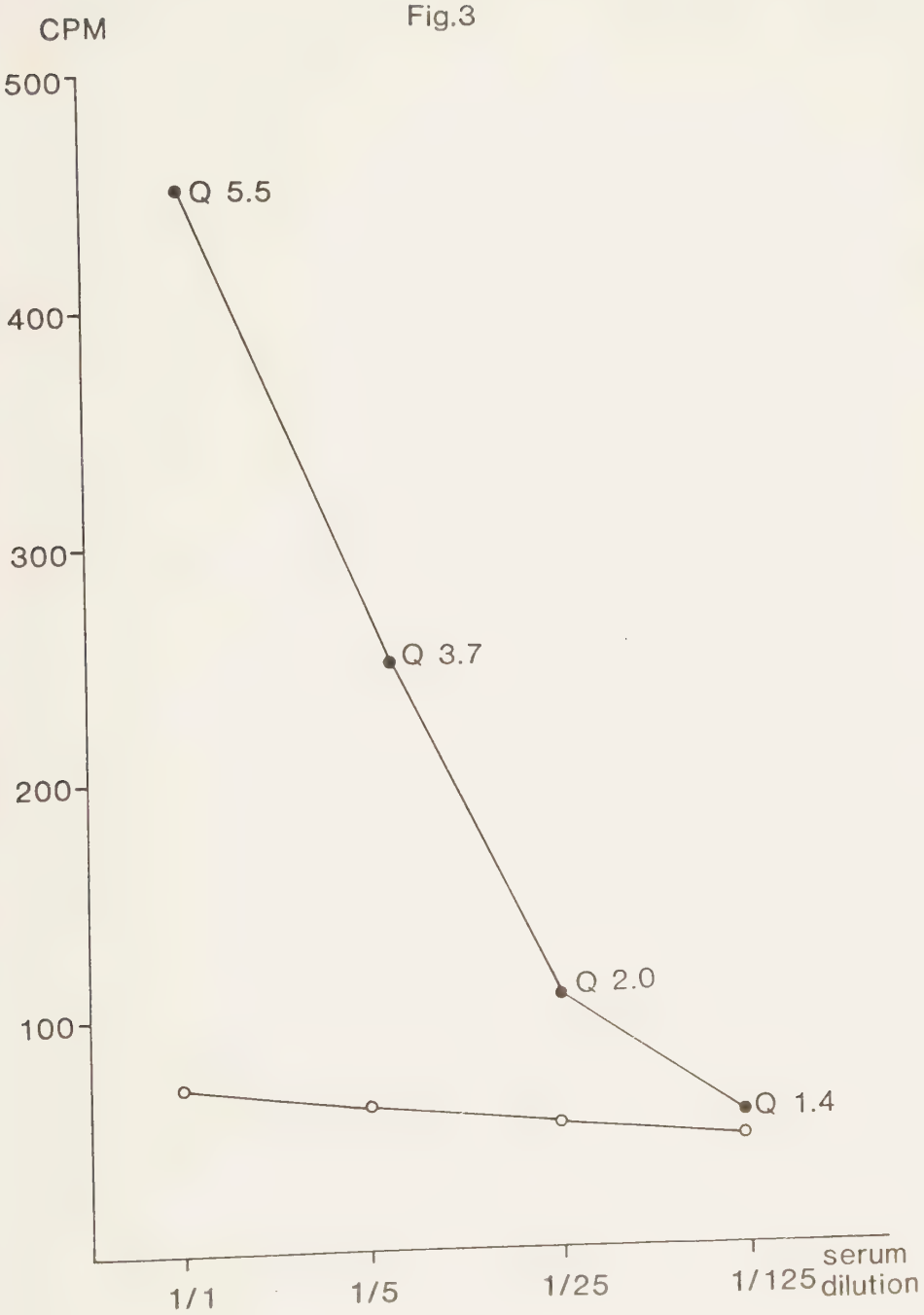

FIGURE 3 Titration of a serum from a multitransfused patient (MT-5, ●—●) against human mononuclear white blood cells from a healthy blood donor (NL-9) as compared to a control serum from a healthy AB blood group donor (AB-29, o—o).

Fig.4

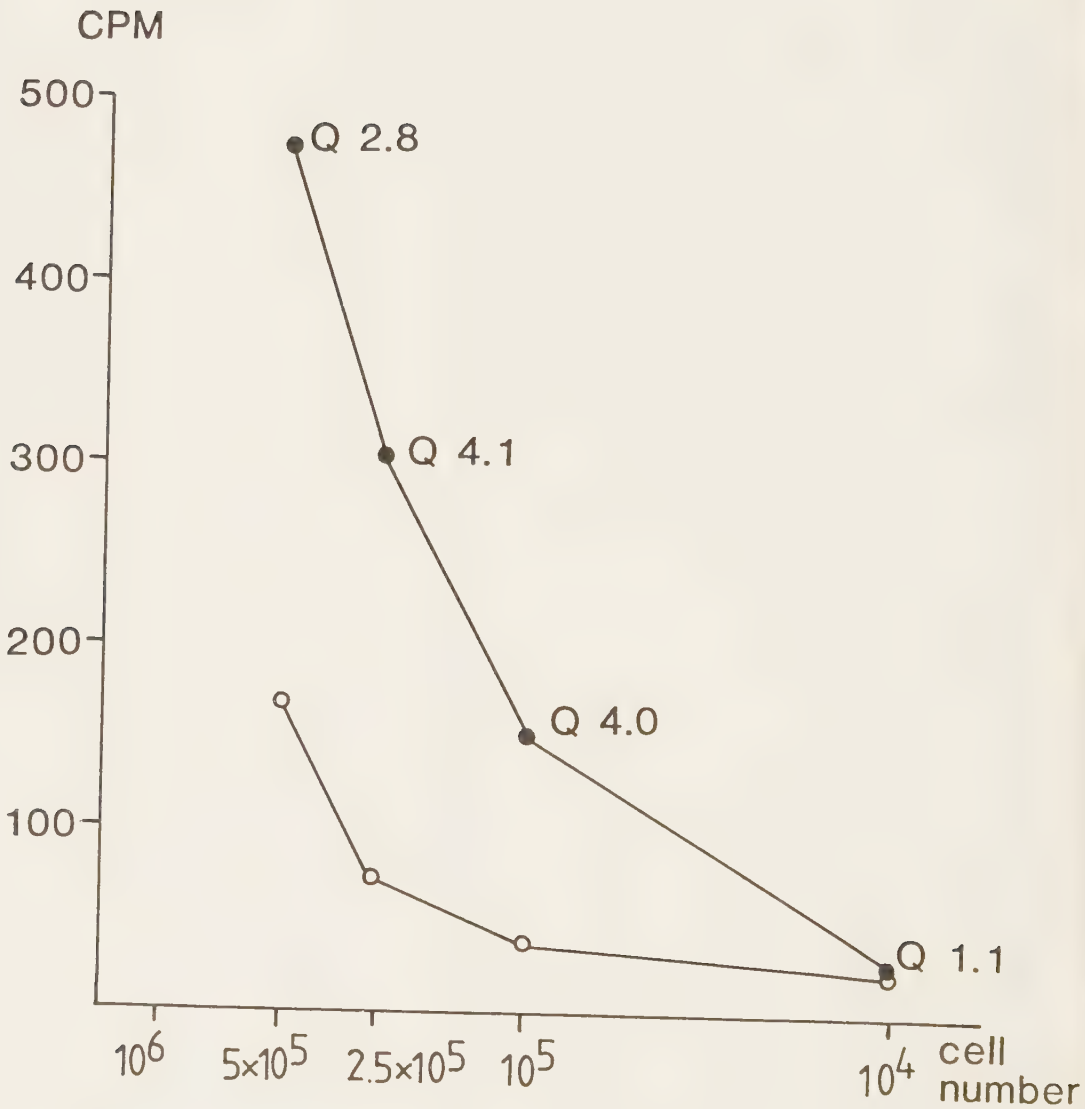

FIGURE 4 Radioactivity uptake by various numbers of target cells (NL-9) with a constant antibody concentration (1:4). Tests of sera from a multitransfused patient (MT-5, ●—●) and a healthy AB blood group donor (AB-29, ○—○).

Fig.5

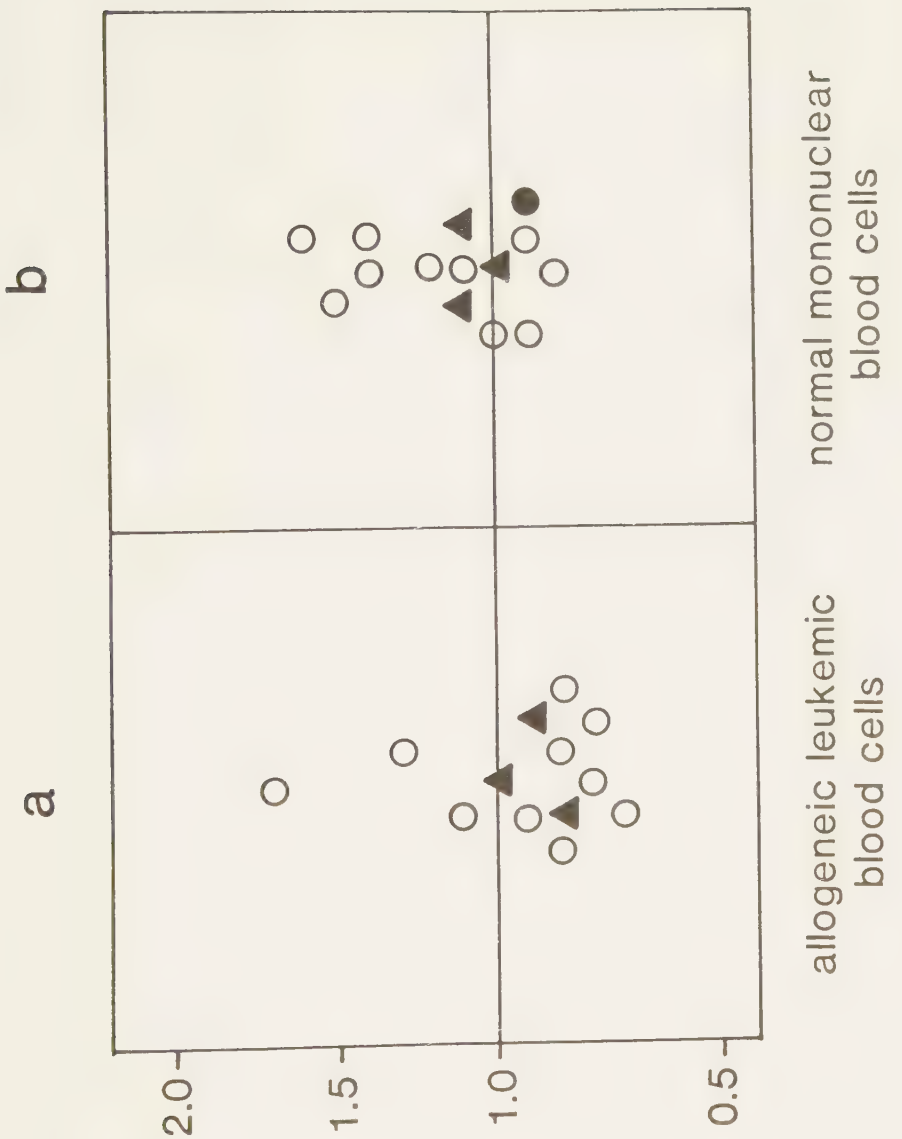

FIGURE 5 Binding of 10 normal AB sera (o—o) to allogeneic leukemic blood cells (a) and 10 normal AB sera and 1 autochthonous serum to normal mononuclear blood cells (b) as compared to 3 reference control AB sera (▲—▲).

Fig.6

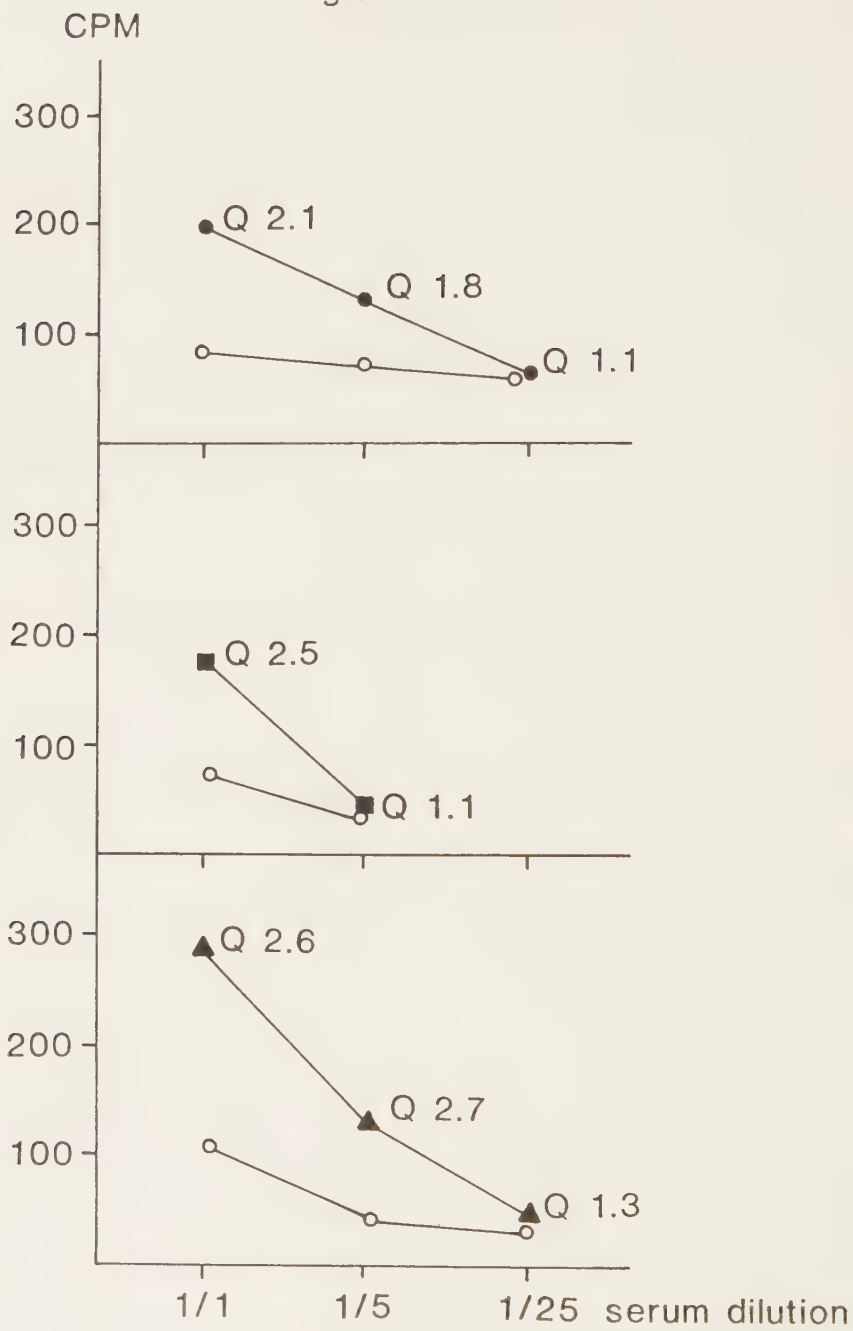

FIGURE 6 Titration of sera from 2 patients with ALL (BL-1A, ●—● ; PC-79, ▲—▲) and a serum from 1 patient with AML (PF-37, ■—■) on autochthonous leukemic target cells as compared to a serum from a healthy AB blood group donor (AB-29, ○—○).

DISCUSSION

Analyses of leukemia-associated markers have mostly utilized monoclonal hybridoma antibodies and a number of different useful markers have been described. Using this technique it was possible to define some cell surface differentiation antigens expressed on T and B lymphocytes, respectively, and data describing unique leukemia- and lymphoma-associated antigens have been presented. The monoclonal antibodies were found to be useful for demonstration of cell markers linked to cell surface antigens and they provide a tool for diagnostic and potentially prognostic correlates in patients with leukemia. These antibodies were predominantely used for characterization of antigens on the surface of lymphoid cells and only a few monoclonal antibodies were described as reactive with membrane markers of myeloid cells. A serious limitation of markers defined by monoclonal antibodies is that they usually are not antigenic in autochthonous systems. Consequently they are not involved in antitumor responses of patients.

Analyses of normal and neoplastic leukocytes were also performed with xenogeneic, specifically absorbed antisera and a number of recently published reports describe antigens specific for human myeloblasts detected by mouse (2), rabbit (4), simian (14) and human (1) antisera used in tests for complement mediated cytotoxicity and indirect immunofluorescence. Strong anti-human leukemia antisera were also raised in rabbits immunized with purified human thymus leukemia-associated antigen. These sera expressed high specificity without any absorption being cytotoxic for leukemic T-cell lines byt not for nonmalignant B-cell lines, a leukemic non-T, non-B-cell line, a leukemic pre B-cell line, normal peripheral blood lymphocytes and T-cells isolated from peripheral blood lymphocytes (15). Heteroantisera were found to be extra-

ordinarily useful for characterization of subpopulations of lymphoid and myeloid cells. However, significant technical difficulties were generally encountered in their preparation and it was difficult to reproduce antibodies with identical specificity.

We have previously presented evidence for the appearance of anti-leukemic antibodies in adult acute leukemia patients reactive with the patients own leukemic cells as demonstrated by ^{51}Chromium-release techniques (6, 7). A binding assay using ^{125}I protein A to detect also specific non-cytotoxic antibodies in acute leukemia has now been established. Important limitations of earlier presented modifications of ^{125}I protein A binding assays have been high background levels of radioactivity testing sera at low dilutions and lack of sensitivity. Optimal laboratory conditions for our assay were established using a rabbit antiserum to human cells and sera from non-leukemic multitransfused patients. Background radioactivity was brought down to very low levels thereby increasing the sensitivity of the assay. It was found that 2/5 sera from multitransfused patients contained antibodies to allogeneic peripheral white blood cells from a healthy blood donor, most probably due to HLA-sensitization. Parallel testing of one of these sera using conventional HLA testing technique (11) showed that our ^{125}I protein A binding assay is considerably more sensitive than the conventional HLA testing method and thus indicates that it may be useful for detection of weak antileukemic antibody responses.

Using bone marrow and peripheral white blood cells harvested from patients with acute leukemias in the acute and remission stages of the diseases, we could demonstrate significant binding of autochthonous sequential sera exclusively to the leukemic cells. These results are analogous to our previously reported findings studying cytotoxic antibodies in patients with AML (6, 7). Also in these studies serum reactivity was usually found to be highest in sera taken from

the patients in partial or complete remission after successful chemotherapy. Our interpretation of these findings is that specific antibodies are bound to tumor cells and soluble tumor antigen in the acute phase of the disease and by reducing the tumor burden through chemotherapy these antibodies are left in excess and detectable in the sera. This anticipates that the antibody producing cells are not seriously damaged by the therapy. Indirect evidence for the presence of specific immune complexes in sera of patients with AML containing potentially cytotoxic antibodies detectable in complement-dependent tests has been previously presented (8).

The fact that the antibodies in patients´ sera only bound to autochthonous leukemic cells and not to autochthonous remission cells is strong evidence for specificity. In 2/3 cases the binding seemed to be higher to autochthonous leukemic bone marrow cells than to peripheral leukemic blood cells and in one case there was no significant binding to leukemic target cells obtained from peripheral blood. One explanation for this is that only diluted serum was used (1:4) and testing undiluted serum might have given significant reactivity with the latter cells.

No correlation was found between the number of blast cells in bone marrow or peripheral blood and the reactivity of sera binding to the target cells. Patient 49 with 85% blast cells in the blood and patient 79 with 4% blast cells in the blood were tested and sequential sera showed the same degree of binding to their own leukemic target cells. Analogous results were found by Halterman et al (10) testing the reactivity of a heterologous antileukemic serum on target cells from peripheral blood of patients with acute leukemias.

No correlation was found between the levels of immunoglobulins in patients' sera and the specific binding to autochthonous target cells obtained from the acute stage of the leukemia. Sera with high concentrations of IgG as PD-60 and PA-63 bound significantly only to autochthonous leukemic target cells but not to autochthonous remission cells (PA-63) or allogeneic target cells (PD-60). This gives further support for the specificity of the binding of patients' sera to their own leukemic cells and argues against non-specific cellular uptake of immunoglobulins.

Serum from a leukemia patient (PC-79) and serum from a multitransfused patient with bone marrow insufficiency (MT-5C) bound to 1/2 and 2/3 allogeneic target cells, respectively. This probably reflects HLA-sensitization since patients with acute leukemia will get several blood transfusions containing a considerable portion of white blood cells. These findings illustrate the importance of testing sera against autochthonous target cells to demonstrate the presence of specific antibodies. In allogeneic combinations there are always interpretation difficulties due to the possible presence of antibodies against blood-group antigens and other antigens.

The titer of the antileukemic antibodies demonstrated with our ^{125}I binding assay was low similarly to the previously studied cytotoxic antibodies (6,7). Significant activity was demonstrated only in undiluted sera or in sera diluted up to the dilution 1:5. One explanation of this may be that cellular immunity is the dominant immunologic defense mechanism in leukemia and that the production of specific humoral antibodies is low.

It is also possible that intensive chemotherapy depresses the production of such antibodies. However, this is not supported by the determination of total immunoglobulins in the patients' sequential sera, which with few exceptions demonstrated only small or moderate depression of the immunoglobulin concentrations.

There are recent reports on the appearance of serum antibodies in the course of such immunotherapy that prolongs remission and survival times in patients with acute leukemia (9). However, allogeneic material was used in these experiments and the specificity of the antibody response must therefore be questioned. In combination with assays for complement dependent cytotoxicity and possibly ADCC assays the established binding assay has considerable potential as a tool for analysis of antileukemic humoral antibody responses in patients with leukemia. Such analysis seems particularly important in connection with various immunologic manipulation procedures such as treatment with immunostimulants, immunization with antigenic material and plasmapheresis but may also be of interest in chemotherapy.

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A NEW APPROACH TO DETECTION OF ANTIGEN-ANTIBODY COMPLEXES BY MICROCALORIMETRIC MEASUREMENTS OF HEAT PRODUCTION IN BLOOD CELLS

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Using batch microcalorimeters of the thermopile conduction type, heat production was measured in human blood in the presence of defined antigens and their specific antibodies. A large increase in heat production, reflecting increased metabolic activity in the blood cells, was found when antigens and specific antisera at certain antigen-antibody proportions were mixed with the blood. The reproducibility and specificity of the method were found to be very good. The present results indicate that calorimetry may be a useful technique for detection of antigen-antibody complexes.

INTRODUCTION

There is growing clinical interest in studying circulating immune complexes and their possible role in the etiology and pathogenesis of disease, especially in the rheumatic diseases and in human neoplasms. Several laboratory methods for detecting antigen-antibody complexes have been introduced, most of them based on the participation of complement in the reactions involved (Barnett et al., 1979). The results obtained using these methods are conflicting and correlate poorly with the degree of activity of the immunological diseases investigated and therefore have limited value for predicting disease exacerbations.

It has been reported that antigen-antibody complexes increase the oxygen uptake by human blood (Strauss and Stetson, 1960) and activate neutrophils (Rossi et al., 1970; Henson, 1974). Clarke et al. (1978) have recently used the measurement of neutrophil aerobic metabolism as a means of detecting immune complexes in a defined experimental system. In systemic lupus erythematosus (SLE) Levin and Thomasson (1974) found increased leucocyte heat production when leucocytes in plasma were exposed to homogenates prepared from autochthonous white blood cells. The investigators

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suggested that this stimulation of leucocyte metabolism was perhaps due to the presence of antigen-antibody complexes.

In other calorimetric studies large increases in heat production were obtained when aerobic metabolism was stimulated in both erythrocytes (Levin, 1973a; Monti and Wadsö, 1976) and leucocytes (Levin, 1973b).

In the exploratory calorimetric experiments reported by Johns et al. (1974) several antigen-antibody reaction systems involving living cellular material were investigated. However, in that work attention was focused on the thermochemistry of the immunological binding processes but possible changes in the metabolic heat production of the cells were not considered.

In the present investigation we have measured heat production in whole venous human blood in the presence of defined antigens and their specific antibodies. The aim of this study was to establish a microcalorimetric method for detecting antigen-antibody complexes and for studying the proportions of antigens and antibodies involved in complex formation.

MATERIALS AND METHODS

Blood

Blood was obtained from volunteer outpatients at the Department of Internal Medicine. Venous blood was collected into 10 ml Vacutainer tubes containing 143 USP units of sodium heparin. After collection, the blood was kept at room temperature for 1 h before calorimetric measurements were performed.

Antigens

Ovalbumin, OA (egg albumin), purchased from Sigma, St. Louis, MO, U.S.A., Lot No. A-5503, was used. The crystallized and lyophilized powder was dissolved in sterile saline before use. For the in vitro tests a stock solution of 3 mg ovalbumin/ml saline, giving equivalence zone proportions with the undiluted specific antiserum, and its 4-fold dilutions in saline were used.

Human albumin, HSA, purchased from AB Kabi, Stockholm, Sweden, Lot No. 51320, as lyophilized powder, was dissolved in sterile saline at a concentration of 3 mg/ml. This stock solution and its 4-fold dilutions in saline were used in the tests.

Antisera

Anti-ovalbumin serum was obtained from rabbits immunized by subcutaneous injection of 1 mg/ml ovalbumin in saline and 1 ml Freund's complete adjuvant twice with an interval of 6 months. The serum was harvested 1 week after the second immunization.

Anti-human albumin serum was obtained from a rabbit immunized using

1 mg/ml human serum albumin in saline and 1 ml of Freund's complete adjuvant injected subcutaneously. The immunization procedure was repeated at 6 and 15 months and the serum was harvested 1 week after the last booster dose.

The sera were stored at −20° C and were heat-inactivated at 56° C for 30 min before use. Immediately before calorimetric experiments the immune complex was prepared by mixing 1 ml of serum (undiluted and at various dilutions) with the specific antigen dissolved in 1 ml of saline.

Calorimetric measurements

The calorimetric measurements were performed with LKB 10700-2 batch microcalorimeters. These instruments are twin calorimeters of the thermopile conduction type with a time constant of 130 sec. The two reaction vessels are each surrounded by a thermopile and the assemblies are enclosed by a metal block. The vessels are made from 18 carat gold and each has two compartments keeping the reagents separated before the process is started. Mixing of the reagents is achieved by rotation of the calorimetric block.

In the experiments reported here, one of the vessels (the reaction vessel) was charged with 4 ml + 2 ml of reagents. The other vessel (the reference vessel) was charged with water. The calorimeter was allowed to equilibrate for 2 h before the mixing process was started.

In the immunological activation experiments, 4 ml of whole blood was mixed with 2 ml of serum preparation containing antigen and specific antiserum at various dilutions. In control experiments 4 ml of whole blood was mixed with 2 ml of serum preparation without specific antibodies or with 2 ml of saline.

By using two calorimeters, activation experiments and corresponding control experiments were conducted simultaneously using the same blood sample.

The signal recorded in the calorimetric experiments is the differential voltage from the two thermopiles. In the present experiments no heat was produced in the reference vessel (except for frictional heat during the mixing process which, however, was essentially cancelled by the corresponding thermal power generated in the reaction vessel).

RESULTS

Figs. 1 and 2 show the calorimetric signal as a function of time (power-time curve) from a typical activation and control experiment, respectively. The initial baseline corresponds to the basal heat production in the blood sample, ca. 250 μW (Bandmann et al., 1975). At time zero the mixing was initiated. This was accompanied by a complex pattern of the calorimetric signals due to differential mechanical effects in the two reaction vessels, dilution of the reagents, and various binding processes between the cells and

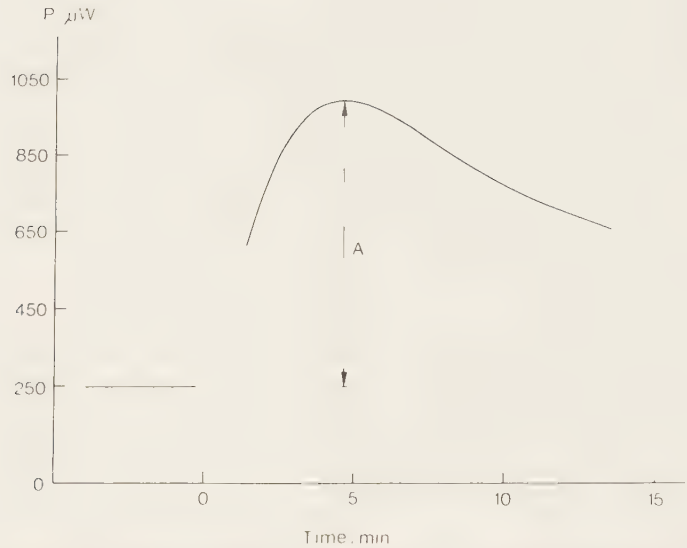

Fig. 1. Typical calorimetric curve from an activation experiment. Mixing took place at time zero. P is the thermal power (not corrected for the thermal inertia of the instrument). The complex calorimetric signal immediately after the mixing is not shown. A represents the activation of the metabolism of the blood cells.

the reagent mixture. This part of the calorimetric signal is not shown.

After this phase the calorimetric signal increased reflecting an increased rate of heat production in the blood cells. In the activation experiments, very high maximal rates were obtained 5—15 min after the mixing of blood and preformed complexes. Due to the thermal inertia of the instruments, the recorded voltage signal was not directly proportional to the thermal power produced in the reaction vessel except at the point were the slope is zero: A in Fig. 1 (Randzio and Suurkuusk, 1980). In the present work we have taken this value as an arbitrary measure of the activation of the blood cell metabolism. (This value was lower than the maximal power value.)

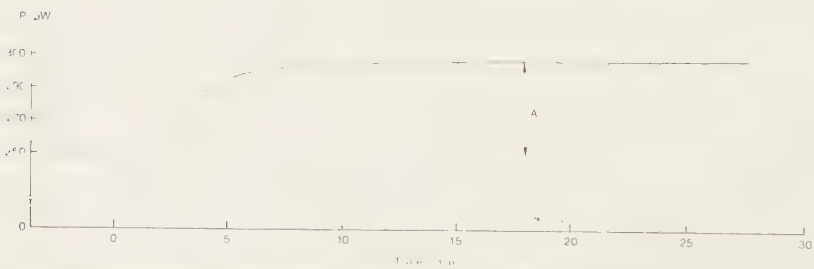

Fig. 2. Calorimetric curve from a typical control experiment where blood was mixed with a preparation of antigen and rabbit serum.

In the control experiments (Fig. 2), a small, but normally essentially constant increase in the heat production level was observed following the mixing process. (As the signal in this case changed only very slowly, it is directly proportional to the increased rate of heat production of the blood cells.)

Blood heat production was in all cases found to increase markedly when preparations of antigen and specific antiserum at certain antigen-antibody proportions were mixed with the blood. The reproducibility of the method was found to be excellent despite the fact that the blood used in the experiments was obtained from different donors (Table 1). Fig. 3 shows the calorimetric antigen titration curve obtained in experiments where blood (4 ml) was mixed with preparations containing antigen-antibody complexes (2 ml). The latter were made up from OA (1 ml, dilutions as indicated in Fig. 3) and undiluted rabbit antiserum (1 ml). Results of control experiments are also given. In these the blood was mixed with preparations consisting of the same anti-serum as was used in the main experiments and an unrelated antigen (HSA).

Fig. 4 shows a corresponding calorimetric titration curve obtained when blood was mixed with preparations consisting of HSA in different dilutions and undiluted rabbit antiserum. Results of control experiments with unrelated antigen (OA) are also shown.

For both titration curves shown in Figs. 3 and 4, maximal values were

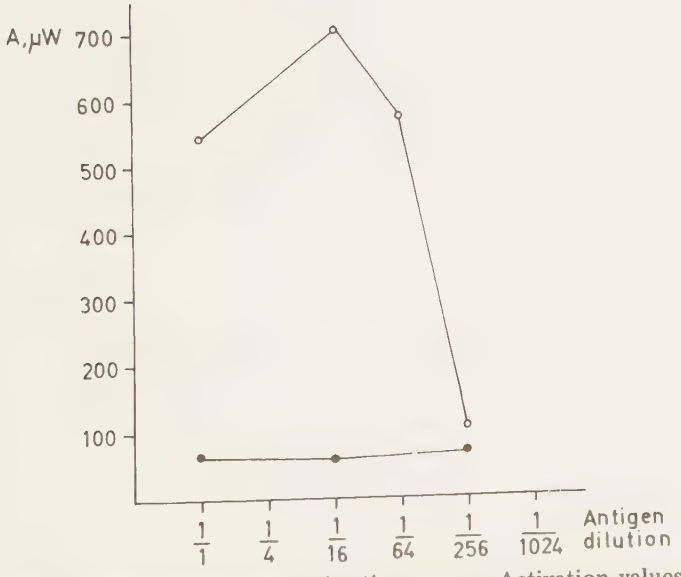

Fig. 3. Calorimetric antigen titration curves. Activation values, A (see Fig. 1), for human blood after mixing with preparations of undiluted OA rabbit antiserum mixed with OA (○———○), or with HSA (●———●) in the dilutions indicated.

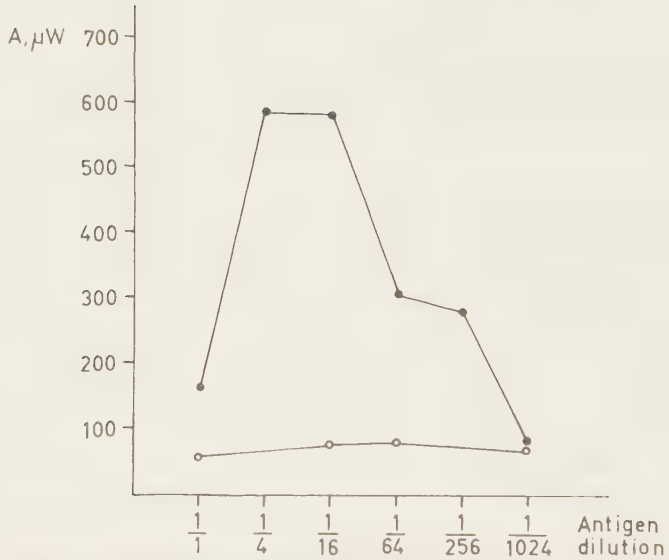

Fig. 4. Calorimetric titration curves. Activation values, A (see Fig. 1), for human blood after mixing with preparations of undiluted HSA rabbit antiserum mixed with HSA (●——●) or with OA (○——○) in the dilutions indicated.

observed at antigen dilutions 1 : 4—1 : 16. The activation values obtained in the control experiments were in all cases significant but far lower than those found when blood was mixed with preparations of antiserum and corresponding antigen in optimal concentration.

A few additional control experiments were also performed where blood was mixed with preparations consisting of rabbit serum and saline. Also in these cases significant but small activation values were found: 50—130 μW. When blood (4 ml) was mixed with preparations (2 ml) consisting of antigen (OA, HSA) and saline, activation values of ca. 40 μW were regis-

TABLE 1

Reproducibility of the activation values, A (see Fig. 1), when 4 ml of human blood from different donors were exposed to 2 ml of various antigen-antibody preparations.

Antigen and its dilution	Antiserum and its dilution	Number of expt.	Activation values ± S.D. (μW)
OA 1 : 1 [a]	OA 1 : 1	4	514 ± 67
OA 1 : 64	OA 1 : 64	2	169 ± 14
HSA 1 : 4 [b]	HSA 1 : 4	2	87 ± 35
HSA 1 : 32	HSA 1 : 4	3	235 ± 43

[a] OA: ovalbumin.
[b] HSA: human serum albumin.

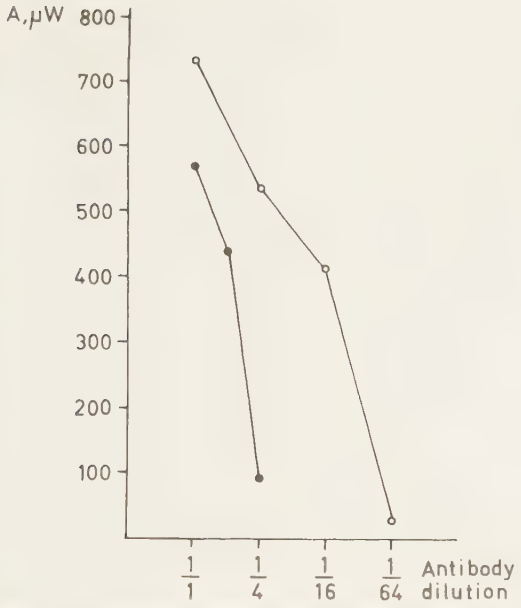

Fig. 5. Activation values for human blood, A (see Fig. 1), when exposed to preparations of OA (○———○) or HSA (●———●) at dilutions 1 : 16 and 4-fold serial dilutions of corresponding antisera.

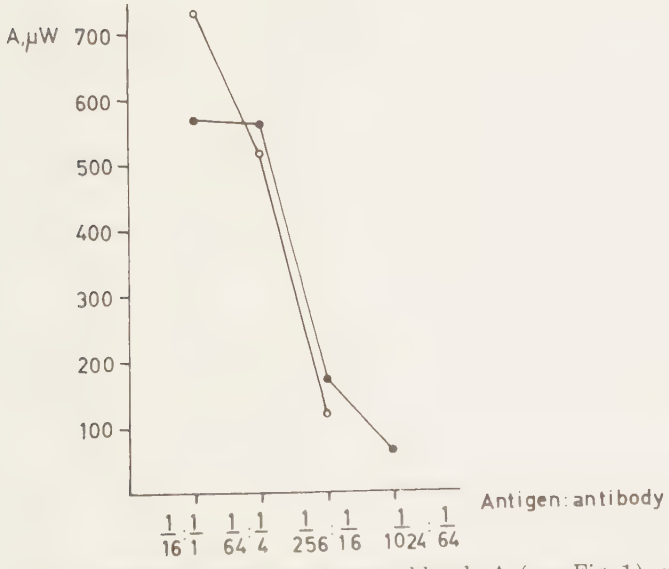

Fig. 6. Activation values for human blood, A (see Fig. 1), when mixed with combined OA/anti-OA serum (○———○) or HSA/anti-HSA serum (●———●). The measurements were performed as a 4-fold serial dilution experiment keeping the mixtures of antigen-antibody in constant proportion 1 : 16.

tered. When blood (4 ml) was mixed with pure saline (2 ml), a still smaller activation value was found: ca. 20 μW. If plasma (4 ml) was mixed with serum diluted with an equal amount of saline there was no heat production observed after the initial small burst of heat evolved at the mixing process.

Fig. 5 shows results of activation experiments where antigens (HSA, OA) at the constant dilution 1 : 16 were tested with specific antisera at serial dilutions. The antigen dilution 1 : 16 was chosen since it was found to give maximal activation in the antigen titration experiments as reported in Figs. 3 and 4. The anti-HSA titration curve shows that the activation was decreased to the values obtained in control experiments at the dilution 1 : 4. With anti-OA serum higher dilutions were required for the activation to decrease to a low value.

The maximal activation value was found for both antigen-antibody-systems when the antigen was diluted 1 : 16 in relation to the specific antiserum (Figs. 3 and 4). Fig. 6 summarizes results of activation experiments where this proportion between antigen and antibody was kept constant whereas the antigen-antibody preparation was serially diluted with saline. A gradual decrease in the activation values was found for increasing dilutions.

DISCUSSION

In the present investigation a calorimetric method for detecting antigen-antibody complexes has been demonstrated. During well defined experimental conditions, antigens and their corresponding specific rabbit antisera, were found to activate the metabolism of human blood cells. The observed maximal increase in heat production, A (see Fig. 1), was very large — 150—180 μW/ml of blood, a range which may be compared with the normal basal heat production in human blood, ca. 60 μW/ml (Bandmann et al., 1975). As shown by results of control experiments the specificity of the measurement was excellent. It may be noted that a small but significant activation of the blood cells was observed also in the different types of control experiments performed. The origin of this effect is unknown.

In the titration experiments there was a limited range in proportion between antigen and antibody required for maximal activation probably corresponding to the zone of equivalence in the formation of antigen-antibody complexes. Dilution of the antigens to about 1 : 16 relative to the corresponding antisera was found to give maximal activation, whereas antigen or antibody in excess gave lower values. This effect of the variation in the proportion of antigen relative to antibody may explain many of the conflicting results earlier found with other assay methods.

Circulating antigen-antibody complexes have been demonstrated in many diseases such as systemic lupus erythematosus and other rheumatic diseases, various human neoplasms, glomerulonephritis and some infectious diseases. However, the significance of circulating immune complexes in the etiology

and pathogenesis of these diseases has not been clarified. In some instances they may merely reflect an immune reaction and the circulating antigen-antibody complexes may be the result of leakage into the circulation of products from a local pathological process. In some situations the presence of circulating antigen-antibody complexes has been found to correlate with the disease activity but, generally, a poor correlation has been noted in studies where certain assay methods were used, presumably because of differences in sensitivity of the methods for detecting complexes with different antigen-antibody proportions.

The method presented here has been shown to give excellent results with respect to sensitivity, specificity and reproducibility in well defined systems. However, the utility of this method for detecting antigen-antibody complexes formed in vivo remains to be shown before the clinical value of the method can be evaluated.

From a practical point of view the method presented in this work is far from optimal. The microcalorimeters employed were mainly designed to meet the requirements of precise thermodynamic investigations and their handling is time consuming. The total time required for one experimental cycle was about 4 h. It should be possible to reduce the measurement time by at least an order of magnitude and the quantities of material could also be reduced significantly. Development work in this direction is in progress as well as further studies to define the nature of the immunological activation process, including the type(s) of blood cells involved and the effect of antigen-antibody complexes formed in vivo. Preliminary results indicate that granulocytes are responsible for the observed increase in blood heat production in the presence of antigen-antibody complexes.

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Immunology 1982 **46** 189

Heat production in different populations of human blood cells exposed to immune complexes *in vitro*: the importance of the Fc parts of immunoglobulins and the influence of active complement

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Summary. By use of a batch microcalorimeter of the thermopile type, heat production was measured in isolated populations of human peripheral blood cells exposed to defined immune complexes formed *in vitro*. It was found that most of the heat production recorded in whole blood after admixture of immune complexes occurs in the granulocytes. Under these conditions small but constantly higher activation values were found in the absence of active complement. It was shown that complexes consisting of antigen and $F(ab)_2$ fragments prepared from the specific antibodies were able to initiate heat production in the cells only in the presence of active complement. These experiments indicate that immune complexes are able to induce increased heat production in the cells either by binding to Fc receptors or by activation of complement through the alternative pathway and subsequent binding of the generated C3b to C3b receptors on the heat-producing cells.

INTRODUCTION

Circulating immune complexes (CIC) play a significant role in the pathogenesis of several diseases (WHO, 1977). Various methods have been used for detection of CIC in attempts to correlate their presence with disease activity. Much effort has been spent on monitoring fluctuations of CIC resulting from drug therapy and plasmapheresis in the management of some clinical disorders (Barnett, Knutson, Abrass, Chia, Young & Liebling, 1979). In a recent study we have described a microcalorimetric method for detection of antigen–antibody complexes (Monti, Fäldt, Ankerst & Wadsö, 1980). Large heat production was registered *in vitro* when whole venous blood was mixed with *in vitro* formed antigen–antibody complexes at certain proportions between antigens and their specific antibodies. However, the blood cells responsible for heat production were not identified. In the present investigation isolated populations of human blood cells are analysed for heat production when mixed with defined antigen–antibody complexes. The results show that granulocytes represent the main source of heat production in whole venous blood when exposed to the complexes.

Since it is known that receptors for C3b and the Fc portion of immunoglobulins are present on human granulocytes (Henson, 1969; Messner & Jelinek, 1970; Ishizaka, Tomioka & Ishizaka 1970; Mantovani, 1975; Ehlenberger & Nussenzweig, 1977), monocytes (Lo Buglio, Cotran & Jandl, 1967; Huber, Polley, Linscott, Fudenberg & Müller-Eberhard, 1968; Huber, Douglas & Fudenberg, 1969) and various

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types of lymphocytes (Lay & Nussenzweig, 1968; MacLennan, Loewi & Howard, 1969; Theofilopoulos, Dixon & Bokisch, 1974), our interest in the present study was also to elucidate the significance of these receptors in initiating heat production in blood cells exposed to immune complexes.

MATERIAL AND METHODS

Cell suspensions

Blood. Blood was obtained from volunteer outpatients and healthy blood donors at the Department of Internal Medicine. Heparinized whole venous blood was collected and handled as previously described (Monti *et al.*, 1980). The various purified blood cells were prepared from the blood and resuspended in autologous plasma, serum or saline. Cell suspensions were collected into polystyrene tubes.

Granulocytes. Granulocytes were prepared by layering leucocyte-rich plasma onto Ficoll-Isopaque (Lymphoprep®, Nyegaard, Oslo, Norway; specific gravity 1·077). After centrifugation for 10 min at 70 ***g*** followed by 15 min at 700 ***g*** the sedimented granulocytes in the bottom of the tubes were washed once and resuspended in autologous plasma or serum and used in the experiments. The suspension contained approximately 90% granulocytes.

Unfractionated mononuclear cells. Unfractionated mononuclear cells consisting of lymphoid cells and monocytes, were collected from the interphase after identical density gravity centrifugation as described above.

Monocyte-depleted mononuclear cells. Monocyte-depleted mononuclear cells, i.e. lymphoid cells, were prepared by removing the monocytes using a magnetic separation procedure. Unfractionated mononuclear cell suspensions were incubated with iron powder (Merck, Darmstadt, West Germany) at 37° under rotation for 30 min and the phagocytic cells were removed with a magnet.

Erythrocytes. Samples of erythrocytes were prepared according to the gel adsorption method described by Nakao, Nakayama & Kankura (1973). The purified cells were suspended in autologous plasma which was made cell-free by centrifugation at 3000 ***g*** for 15 min. Cell concentration was adjusted to a haematocrit of about 40%.

Platelets. Unless otherwise indicated venous blood samples were collected into 10 ml Vacutainer tubes containing 25 USP units of sodium heparin and 2 ml of a 5% Dextran-80 solution (Pharmacia, Uppsala, Sweden). Platelet rich plasma (PRP) was prepared using the method described by Levin (1971). In two experiments venous blood was collected into 10 ml Vacutainer tubes containing 2 ml of 3·8% sodium citrate with 0·2 mg potassium sorbate and centrifuged at 150 ***g*** for 15 min. The supernatant, free of leucocytes and erythrocytes but containing the platelet fraction, was used for these calorimetric experiments. Duplicate platelet counts were made by phase microscopy before each calorimetric measurement.

Antigens

Ovalbumin. Ovalbumin (egg albumin) purchased from Sigma, St Louis, U.S.A., Lot Number A-5503, was used. The crystallized and lyophilized powder was dissolved in sterile saline. For the *in vitro* tests a stock solution of 3 mg ovalbumin/ml saline, giving equivalence zone proportions with the undiluted specific antiserum, was prepared.

Human albumin. Human albumin (HSA), purchased from AB Kabi, Stockholm, Sweden, Lot Number 51320, as lyophilized powder, was dissolved in sterile saline at a concentration of 3 mg/ml.

Anti-ovalbumin serum. Anti-ovalbumin serum was prepared by immunization of a rabbit subcutaneously twice with 1 mg ovalbumin in 1 ml saline and 1 ml Freund's complete adjuvant at an interval of 6 months. The serum was harvested 1 week after the second immunization. It was stored at −20° before use. In some experiments complement was inactivated by heating at 56° for 30 min.

$F(ab)_2$ fragments. $F(ab)_2$ fragments were prepared from anti-ovalbumin serum. Immunoglobulin was precipitated from 5 ml of anti-ovalbumin serum with 40% saturated ammonium sulphate. After dialysis against 0·1 M sodium acetate, pH 4·5, and determination of the protein concentration (25 mg/ml) with a modified Folin method (Lowry, Rosenbrough, Fan & Randall, 1951), pepsin (Worthington, New Jersey, U.S.A., 3000 u./mg) was added at an enzyme : substrate ratio of 1 : 20

and the sample was incubated at 37° for 18 hr. The digestion was stopped by dialysis against phosphate-buffered saline, pH 7·2 (0·12 M NaCl, 0·03 M phosphate). The sample was then filtered on a Sephadex G-200 column (Pharmacia Fine Chemicals, Uppsala, Sweden, 2·5 × 90 cm, elution rate 18 ml/hr, fractions consisting of 4·5 ml) and the fractions of the second peak, which contained IgG as determined in double diffusion test in gel using porcine antiserum to rabbit IgG (Dako, Copenhagen, Denmark), were pooled and concentrated to 30 mg/ml.

Antigen–antibody complexes

Immune complexes were formed with ovalbumin diluted 1:16 as compared with the stock solution and anti-ovalbumin serum or its $F(ab)_2$ preparation diluted 1:4. The proportions used between antigens and antibodies, giving high activation values, were chosen on the basis of our previous calorimetric experiments (Monti *et al.*, 1980). One millilitre of the antigen solution was mixed with 1 ml of antiserum and the resulting 2 ml, containing immune complexes, were used in the experiments.

Calorimetric measurements

Pairwise calorimetric measurements were performed simultaneously with two twin LKB 10700-2 batch microcalorimeters. The two reaction vessels are each surrounded by a thermopile. Each vessel has two compartments keeping the reagents separated before the process is started. In the experiments reported here the reaction vessels were charged with 2 ml of solutions containing antigen–antibody complexes and 4 ml of plasma, whole blood, washed blood cells in saline or suspensions of purified populations of blood cells. The reference vessels were charged with water. Mixing of the reagents was achieved by rotation of the calorimetric block. The signal recorded is the differential voltage from the two thermopiles. The initial baseline (Fig. 1) corresponds to the basal heat production in the blood sample, about 250 μW (Bandmann, Monti & Wadsö, 1975). If the blood cells will be activated following mixing of the two reagents a persisting increase of the calorimetric signal will be found. The maximum value of this increase, normally after 5–10 min, is taken as the 'activation value' for the cell sample. A more detailed description of the calorimetric measurement is given elsewhere (Monti *et al.*, 1980).

RESULTS

Results are summarized in Tables 1, 2, 3 and 4 and Figs 1 and 2.

Figure 1 shows the calorimetric signal as a function of time in a typical activation experiment.

When simultaneous pairwise experiments were carried out comparing activation values in whole blood and purified cell fractions or plasma, almost the same

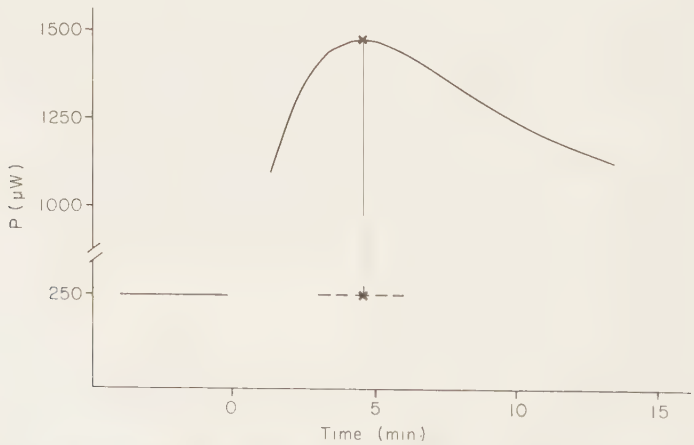

Figure 1. Typical activation experiment. P is the thermal power measured in μW. The initial baseline corresponds to the basal heat production in the blood sample due to its metabolic activity. Mixing of the reagents was performed at time zero. The calorimetric signal (A) represents the heat production generated by the activation of the blood cells in the presence of immune complexes.

Table 1. Results of simultaneous experiments where activation values were measured when whole blood and a pure cell fraction or plasma, respectively, were mixed with immune complexes composed of OA* and anti-OA serum

Experiment no.		Activation values (μW)
1	Blood	345
	Granulocytes†	348
2	Blood (2·2)	310
	Granulocytes (2·2)	344
3	Blood (1·4)	561
	Mononuclear cells (1·4)	72
4	Blood (1·2)	372
	Mononuclear cells (1·2)	89
5	Blood (haematocrit 40%)	283
	Erythrocytes (haematocrit 40%)	0
6	Blood (haematocrit 40%)	327
	Erythrocytes (haematocrit 40%)	0
7	Blood (265)	510
	Platelets (265)	0
8	Blood (225)	353
	Platelets (225)	0
9	Blood	532
	Plasma	0
10	Blood	332
	Plasma	0

The blood cell fraction investigated in this comparative study had the same cell concentration in whole blood samples and in the pure cell suspensions. The cell concentration ($\times 10^9$/litre) is given within parentheses

* OA, ovalbumin.

† Granulocyte count was not made but according to previous experience with the same cell preparation method, it was estimated to be roughly the same in whole blood and in the granulocyte suspensions.

values were found in whole blood and in the granulocyte suspensions. Small but significant activation values were observed for mononuclear cell suspensions. No activation was noted for erythrocytes, platelets and cell-free plasma (see Table 1). Also when citrate was used instead of heparin in two experiments, no cell activation was noted when the platelets were exposed to the immune complexes. As previously reported (Monti *et al.*, 1980) no significant activation was recorded when antigen alone or antiserum alone were added to blood cells (data not shown).

In another series of pairwise simultaneous experiments, activation values were measured when granulocytes and one other purified cell fraction or plasma were mixed with the immune complexes. Cell suspensions and cell-free plasma were obtained from the same blood samples used to prepare the granulocyte suspensions. Table 2 shows the activation values in plasma and in the different cell fractions compared with the increase of heat production found in the granulocyte suspensions when mixed with the immune complexes.

A linear correlation was found between the number of cells in the granulocyte suspensions and activation values ($r = 0{\cdot}89$, $P < 0{\cdot}001$; (Fig. 2).

The significance of the Fc part of IgG molecules in initiating heat production of the blood cells when exposed to antigen–antibody complexes and the influence of active complement on the reaction was studied. $F(ab)_2$ fragments of rabbit anti-OA serum

Table 2. Results of simultaneous experiments where activation values were measured when granulocytes and one other blood cell fraction or plasma, respectively, were mixed with immune complexes composed of OA* and anti-OA serum

Experiment no.		Activation values (μW)
1	Granulocytes†	407
	Mononuclear cells†	120
2	Granulocytes (2·5)	220
	Mononuclear cells (2·5)	71
3	Granulocytes (1·6)	247
	Mononuclear cells (1·6)	58
4	Granulocytes (2·8)	395
	Mononuclear cells (2·8)‡ (monocyte-depleted)	16
5	Granulocytes (1·8)	368
	Mononuclear cells (1·8)‡ (monocyte-depleted)	53
6	Granulocytes§	167
	Erythrocytes (haematocrit 40%)	0
7	Granulocytes (7·3)	680
	Erythrocytes (haematocrit 41%)	0
8	Granulocytes (6·5)	1·907
	Platelets (194)	0
9	Granulocytes (8·3)	1·569
	Platelets (228)	0
10	Granulocytes (0·9)	98
	Plasma	0
11	Granulocytes (3·5)	383
	Plasma	0

The cell concentration ($\times 10^9$/l) is given within parentheses.

* OA, ovalbumin.

† Granulocyte and mononuclear cell counts were not made but according to previous experience with the same cell preparation methods, the cell concentration was estimated to be roughly the same for the granulocyte and mononuclear cell suspension.

‡ The phagocytic cells were removed with a magnet after incubating the mononuclear cell suspension with iron powder at 37° for 30 min.

§ Due to technical difficulties during the experiment the cell concentration is unknown.

were prepared to determine whether Fc receptors and/or complement receptors on the heat-producing white blood cells are required for the reaction with immune complexes. The results of these experiments are summarized in Table 3. The $F(ab)_2$ preparation at the dilution 1:4 was mixed with OA diluted 1:16, i.e. at the same proportion as the untreated antiserum and OA. An increased heat production was registered in the presence of complexes formed with OA and $F(ab)_2$ fragments of anti-OA only when active complement was present. This effect was not found when these complexes were mixed with washed blood cells suspended in saline. The increased heat production in the presence of active complement was of about the same order of magnitude as that induced by anti-OA–OA complexes when tested in simultaneous experiments and at the same proportions between antigen and antibody. In order to test the specificity of the reaction

Table 3. Experiments demonstrating the importance of the Fc parts of immunoglobulins and/or presence of active complement for increased heat production of blood cells when mixed with immune complexes

Experiment	Antigen*	Antiserum†	Cell suspension	Granulocyte concentration ($\times 10^9$/litre)	Heat production (μW)
1	OA	Anti-OA (+)	Whole blood	NC‡	513
	OA	$F(ab)_2$	Whole blood	NC	579
2	OA	Anti-OA (+)	Whole blood	NC	260
	OA	$F(ab)_2$	Whole blood	NC	233
3	OA	$F(ab)_2$	Whole blood	3·3	499
	HSA	$F(ab)_2$	Whole blood	3·3	78
4	OA	$F(ab)_2$	Whole blood	2·7	401
	HSA	$F(ab)_2$	Whole blood	2·7	79
5	OA	Anti-OA (−)	Washed blood cells in saline	2·8	402
	OA	$F(ab)_2$	Washed blood cells in saline	2·8	0
6	OA	Anti-OA (−)	Washed blood cells in saline	1·8	253
	OA	$F(ab)_2$	Washed blood cells in saline	1·8	0
7	OA	Anti-OA (−)	Washed blood cells in saline	3·8	571
	HSA	Anti-OA (−)	Washed blood cells in saline	3·8	26
8	OA	Anti-OA (−)	Washed blood cells in saline	2·7	320
	HSA	Anti-OA (−)	Washed blood cells in saline	2·7	25
9	OA	Anti-OA (−)	Whole blood	5·5	777
	HSA	Anti-OA (−)	Whole blood	5·5	64
10	OA	Anti-OA (−)	Whole blood	4·5	445
	HSA	Anti-OA (−)	Whole blood	4·5	44

* OA (ovalbumin) and HSA (human serum albumin) were used as antigens at the dilution 1:16.

† Anti-OA (anti-ovalbumin serum) was used unheated (+) and after inactivation of its complement's content by heating to 56° for 30 min (−). $F(ab)_2$ is the $F(ab)_2$ fragment of anti-OA. Anti-OA as well as its $F(ab)_2$ preparation were used at the dilution 1:4.

‡ NC, not calculated.

two different preparations were made, one composed of anti-OA and an unrelated antigen, HSA, and the other preparation composed of anti-OA and OA; activation was then recorded simultaneously pairwise when these two preparations were mixed with blood. Identical experiments were carried out when anti-OA was substituted by its $F(ab)_2$ fragment. The activation values obtained in the control experiments with HSA were in all cases significant but far lower than those found when blood was mixed with preparations of OA and anti-OA serum or its $F(ab)_2$ preparation (see Table 3).

Immune complexes consisting of OA and specific antibodies were able to increase heat production of the cells in the absence as well as in the presence of active complement. Small but constantly higher activation values were registered in the absence of active complement (Table 4).

DISCUSSION

It has been reported that antigen–antibody complexes activate granulocytes (Rossi, Zatti, Patriarca & Cramer, 1970; Henson, 1974) with subsequent stimulation of aerobic metabolism (Clarke, Chassoux &

Table 4. Comparison of heat activation values of blood cells mixed with immune complexes in the presence and absence of active complement

Experiments	Antigen*	Antiserum†	Cell suspension	Granulocyte concentration ($+10^9$/litre)	Heat production (μW)
1	OA	Anti-OA (+)	Washed blood cells in saline	5·5	689
	OA	Anti-OA (−)	Washed blood cells in saline	5·5	1096
2	OA	Anti-OA (+)	Washed blood cells in saline	3·0	516
	OA	Anti-OA (−)	Washed blood cells in saline	3·0	568
3	OA	Anti-OA (−)	Granulocytes in serum	2·5	180
	OA	Anti-OA (−)	Granulocytes in heat inactivated serum	2·5	307
4	OA	Anti-OA (−)	Granulocytes in serum	4·0	433
	OA	Anti-OA (−)	Granulocytes in heat inactivated serum	4·0	495

* OA (ovalbumin) was used at the dilution 1:16.

† Anti-OA (anti-ovalbumin serum) was used unheated (+) and after inactivation of its complement's content by heating to 56° for 30 min (−) at the dilution 1:4.

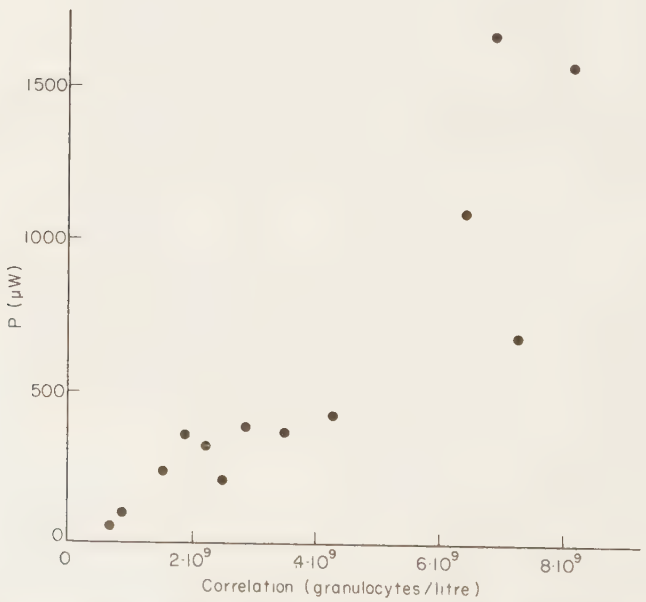

Figure 2. Correlation between the number of cells in the granulocyte suspensions and the activation values ($r = 0{\cdot}89$, $P < 0{\cdot}001$).

MacLennan, 1978). It has also been demonstrated that intact granulocytes phagocytoze antigen–antibody complexes formed *in vitro* (Ward & Zvaifler, 1973). Levin (1973) has previously found that heat production increases in phagocytozing leucocytes. When human granulocytes are exposed to immune complexes activation of oxidative metabolism is registered (Goldstein & Weissmann, 1979). Such metabolic activation is initiated when the cell surface is interacting with the complexes and is independent of phagocytosis of them (Goldstein & Weissmann, 1979).

In a previous study we have described a new microcalorimetric method for detection of immune complexes (Monti *et al.*, 1980). Large increase of heat production was registered *in vitro* when whole venous blood was mixed with defined antigen–antibody complexes formed *in vitro* at certain proportions between antigen and specific antibody. The effector cells responsible for heat production were investigated in the present study. It was found that granulocytes were responsible for the main activation of heat production in the blood. Almost the same activation values were found in whole blood and in the granulocyte suspensions containing a corresponding number of cells. Unfractionated mononuclear blood cells were also activated when the immune complexes were added, but the activation for these cells was significantly lower than that for the same number of granulocytes. After removal of monocytes from these cell suspensions the activation values were further reduced. However, monocyte-depleted mononuclear cell suspensions were still activated when exposed to the complexes. This activation may be generated from a few contaminating monocytes since the iron powder separation procedure does not completely eliminate such cells. Alternatively, the observed effect could be due to stimulation of some of the lymphoid cells left after elimination of the monocytes.

When erythrocytes, platelets or plasma alone were mixed with the complexes no increase of heat production was registered. Since it has been observed that platelets are stimulated metabolically when exposed to immune complexes (Mustard & Packham, 1968), it is surprising that we found no signs of activation for this cell population. The lack of cell activation was probably not due to heparin, since, in fact, also when citrate was used as anticoagulant no activation was obtained. Further studies are in progress to clarify these findings.

Binding of antigen–antibody complexes to granulocytes and subsequent activation of the cells is mediated by Fc and C3b receptors on the cell surface. The presence of Fc receptors (Henson, 1969; Messner & Jelinek, 1970; Ishizaka *et al.*, 1970; Mantovani, 1975; Ehlenberger & Nussenzweig, 1977) and C3b receptors (Henson, 1969; Mantovani, 1975; Ehlenberger & Nussenzweig, 1977) on granulocytes has been demonstrated. Of the four subclasses of IgG, IgG1 and IgG3 appear to bind most efficiently to the Fc receptors on the cells (Messner & Jelinek, 1970).

It is known that C3b can be generated from complement activation by either the classical or the alternative pathway. Complete antibodies of the IgG and/or IgM classes bound to specific antigens are necessary for classical complement activation, whereas $F(ab)_2$-fragments of IgG antibodies bound to antigens in the form of $F(ab)_2$ antigen–antibody complexes are able to generate C3b through activation of the alternative pathway (Gadd & Reid, 1980). With soluble immune complexes it has been reported that complement can act either to increase or decrease the affinity of immune complexes for cell membranes, depending upon the amounts of complement added (Miller & Nussenzweig, 1974). Recently, Erdei, Fust, Medgyesi & Gergeley (1980) demonstrated complement-dependent inhibition of Fc receptors on human peripheral blood mononuclear cells.

Our results strongly indicate that Fc receptors for IgG and C3b receptors are involved in the heat production recorded after admixture of antigen–antibody complexes. Immune complexes formed with OA and $F(ab)_2$ fragments prepared from anti-OA serum did not activate the heat production in the cells in the absence of active complement. In contrast, complexes formed with OA and anti-OA serum, did activate the same cells. However, immune complexes formed with OA and $F(ab)_2$ fragments initiated significant heat production in the cells in the presence of complement, probably as a consequence of complement activation through the alternative pathway and subsequent C3b generation (Gadd & Reid, 1980). In control experiments the same $F(ab)_2$-fragments were mixed with HSA as an unrelated antigen. No heat production of the cells above that obtained with mixtures of untreated antiserum and the same antigen was registered, which demonstrates that the $F(ab)_2$ fragments *per se* do not initiate heat production. The origin of the heat production in these experiments with HSA is unknown, *cf.* Monti *et al.* (1980).

In the present investigation we also registered a small but consistent increase of heat production when cells were reacting with immune complexes in the

absence of active complement as compared with the reactions where active complement was present. Our results are in accordance with previous reports (Miller & Nussenzweig, 1974; Erdei *et al.*, 1980) on the influence of complement on the reactions between Fc receptors of blood cells and antigen–antibody complexes. The biological significance of this effect of complement registered *in vitro* is unknown. It might be a modulator mechanism *in vivo* regulating tissue damage caused by immune complexes, as for instance in the immune complex diseases. This is supported by the reports on SLE-like syndromes in patients with deficiencies in the blood of some complement factors such as C1 and C2 (Klemperer, Woodworth, Rosen & Austen, 1966; Pickering, Naff, Stroud, Good & Gewurz, 1970; Moncada, Noorbibi, Good & Windhorst, 1972).

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